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Editorial

Intraoperative Cytology
Back to the Future?

In 1927, Dudgeon and Patrick[1] published a report of what was then "a new method for the rapid microscopical diagnosis of tumours" and analyzed the results of 200 cases examined by this technique. The publication of this report does not appear to have stimulated any massive reaction, as evidenced by the fact that—according to a recent literature review[2]—only three more communications on this subject were published within the next 25 years. A belated response has been noted in the last two decades, however, with the same review article citing 17 manuscripts published since 1970, to which several more could easily be added. The latest of these is the report of Mair and colleagues that appears in this issue.[3]

In that report, the authors have compared both the degree of diagnostic accuracy and the quality of the specimens obtained in a series of consecutive cases studied by both intraoperative cytology and frozen section. Cases involving the assessment of surgical resection margins were excluded. A unique component of this study was that most of the cytologic examinations were performed by fine-needle aspiration of the specimens, with a smaller proportion examined by scrape, crush, or imprint smears. All cytologic material was alcohol fixed and hematoxylin and eosin stained. Three major conclusions were reached: (1) the quality of cytologic preparations was significantly superior to that of frozen sections; (2) the accuracy of diagnosis by the two techniques was not significantly different; (3) in all but one case, when one technique failed to distinguish a benign from a malignant diagnosis, the other technique was successful. Thus, the authors conclude that "because cytology neither adds significantly to the time needed for F/S preparation nor imposes excessive additional demands on a routine anatomic pathology laboratory (in terms of equipment, stains, and personnel) the authors advocate the simultaneous use of both F/S and cytology in the intraoperative evaluation of surgical specimens."

We strongly endorse this statement and indeed would go beyond it to note that, in many situations, intraoperative cytologic techniques can actually replace, rather than merely complement, the traditional frozen section. Many of the reasons for this conclusion are mentioned by Mair and colleagues and have been cited by us as well in a series of publications on the subject.[4-11] If technical specimen adequacy and diagnostic accuracy are not compromised

by a cytologic preparation—as demonstrated in the series of Mair and associates, in our own experience, as well as that of others[12-16]—then the rationale for using one technique or the other depends on other considerations. One of the major factors involved is time. Mair and colleagues state that "cytology smears in this study required an average of two minutes to prepare, compared with a ten-minute mean time for F/S preparation", and this is our experience as well. The advantage is clear when considering the situation of a busy operating room providing multiple specimens simultaneously. In addition, the time savings (for the pathologist, the surgeon, and the anesthetized patient) is also notable when examining several portions of one specimen. Examples include multiple lymph nodes in a pelvic dissection in a patient with prostatic carcinoma[17] and multiple zones of firm tissue (fat necrosis versus residual carcinoma) surrounding a biopsy cavity in a wider excision or mastectomy for carcinoma.[6] In each of these and similar situations, several cut surfaces to be examined can be imprinted or smeared on the same slide if an air-drying technique is used rather than one requiring immediate fixation.[6,11]

The converse of the large or multiple specimen is the minute specimen that may be used in its entirety in the preparation of a frozen section, leaving no residual tissue for subsequent permanent sections. In this situation as well, an imprint often provides an accurate diagnosis while leaving tissue available for further study. The further study thus facilitated may be not only the microscopic examination of a permanent section but also electron microscopy, hormone receptor assays, flow cytometry, lymphoid cell marker studies, and other adjunctive tests that may be indicated in a particular case.

Certain tissues that often do not permit technically adequate frozen sections also may be appropriate for intraoperative cytologic diagnosis. For example, we have made appropriate diagnoses in the operating room on both fatty and bony specimens, in which frozen section diagnosis would have been difficult if not impossible. Necrotic tissues also are easier to examine and more likely to yield a diagnosis by cytologic techniques.

We perform intraoperative cytology exclusively in cases in which the specimen is submitted from a known human immunodeficiency virus-positive patient or in other situations in which we suspect infection that would result

in contamination of the cryostat. Most of the lesions likely to be seen in this setting (*i.e., Pneumocystis carinii, Mycobacterium avium intracellulare,* and other infections, as well as malignant lymphoma) are diagnosed as accurately, if not more accurately, by cytology than by frozen section,[18] with the exception of Kaposi's sarcoma. We have found this lesion to be particularly difficult to diagnose cytologically, even when it is grossly apparent in a specimen such as a lymph node. In this situation, we ensure that the surgeon is aware that false-negative diagnoses are common in our experience, and to date this policy has not resulted in any major adverse consequences for the patients involved.

A consideration of the consequences to the patient of an incorrect or misleading intraoperative diagnosis is essential to the interpretation of the results of intraoperative consultation. The Association of Directors of Anatomic and Surgical Pathology recently included a statement on intraoperative consultation in its recommendations on quality control and quality assurance in surgical pathology and autopsy pathology.[19] In addition to a statement as to whether each intraoperative diagnosis agrees or disagrees with the final diagnosis or was appropriately or inappropriately deferred, as well as the reason for major disagreement or inappropriate deferral (interpretation, block sampling, specimen sampling, technical inadequacy, lack of essential clinical or pathologic data, or other), the Association also recommended that the medical consequences of the major disagreements and inappropriate deferrals be listed as either none, minor/questionable, or major. Incorrect or inappropriately deferred diagnoses with major implications for patient care obviously need to be analyzed further. This sort of analysis can be applied equally to a comparison of intraoperative cytologic and frozen section diagnoses. For example, Mair and coworkers note correctly that the distinction between a fibroadenoma and fibrocystic changes, or between an infiltrating ductal and infiltrating lobular carcinoma of the breast, may be more difficult with cytologic examination than by frozen section. However, at our own institution, breast biopsies are performed almost exclusively as outpatient procedures, and the rationale for performing intraoperative pathologic consultation on these specimens is to identify malignant tumors for submission of tissue for such studies as hormone receptor assays, flow cytometry, and oncogene amplification studies. Thus, in this setting, a diagnosis of "benign" or "carcinoma" is sufficient, and we see no reason to prefer one technique to another because it is capable of rendering a more specific diagnosis. In other clinical situations, in which a more specific diagnosis is required during operation, we may indeed prefer to perform a frozen section, while in yet other situations (for example, characterizing the cell type

of a malignant lymphoma) intraoperative cytology may even provide a more specific diagnosis than a frozen section. The final message is that we evaluate each case prospectively on its own merits and decide at that time whether to perform a cytologic procedure, a frozen section, both, or neither. If we decide on a cytologic technique, we then have the option of an imprint, a squash preparation, or a scrape and smear, followed by the option of air drying with Diff-Quik staining or alcohol fixation with hematoxylin and eosin staining. We have only rarely used fine-needle aspiration of the intraoperative specimen, and to reduce sampling errors, as noted by Mair, we usually scrape several cut surfaces of the specimen.

Each technique has specific indications and limitations. For example, we now always use air-dried Diff-Quik–stained imprints for the intraoperative evaluation of biopsy specimens in cases of hyperparathyroidism because we have learned that this methodology is better suited to the evaluation of intracytoplasmic lipid, and thus to the level of functional activity of the parathyroid cells (7). On the other hand, we always prepare an alcohol-fixed, hematoxylin and eosin-stained slide when looking at suspected brain tumors because the glial fibrillary network of primary gliomas is better demonstrated by this technique, thus facilitating the differential diagnosis from metastatic carcinoma, meningioma, and other lesions. In examining lymph nodes, we have learned that imprints provide better cellular preservation in high-grade lymphomas but focal lesions such as granulomas and metastases can be detected more effectively by a scrape and smear. Many other such examples might be cited, but the general message here is that intraoperative cytology offers many choices, and the knowledge of when to favor one over another comes only from long experience.

Finally, we would mention that there are several situations in which intraoperative cytology generally is not as useful as frozen section examination. These include the evaluation of resection margins and of the presence or absence of stromal invasion or the depth of invasion of a malignant tumor. In addition, whenever there is a discrepancy between the gross and cytologic appearances of a specimen (for example, when a specimen appears grossly malignant but the cytology is benign, as in some lobular carcinomas of the breast, a frozen section must be performed. If the cytologic material is technically inadequate or borderline (an unusual occurrence both in our experience and that reported by Mair and colleagues), certainly a frozen section should be done as well. Finally, if the surgeon or the patient specifically requests a frozen section rather than a cytologic technique, we will grant that request.

Using these general indications, our recent experience has been that about two thirds of our intraoperative con-

sultations are performed using cytologic techniques only, with an additional 10% based on gross examination. Thus, we actually perform a frozen section in only about one fourth of all of our consultations.[9] The cryostat, of course, remains an indispensable part of our diagnostic armamentarium, but we suggest that, after a sufficient period of performing intraoperative cytology and frozen sections side by side, many pathologists will find, as we have, that the cytologic techniques alone will suffice in most cases.

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Editorial

Parvovirus B19, Hydrops Fetalis, and Fetal Wastage
An Etiologic Sequence

Many unfortunate pathologic testimonials at the surgical pathology bench and autopsy table support the fact that the gravid uterus, which is at least a protected environment metaphorically, may be the site of any number of extrinsic perils to its occupant, the embryo-fetus. In the last 20 years, Naeye, in a series of clinicopathologic and epidemiologic studies, has shown very convincingly that the health of the baby is a reflection of the overall health and welfare of the mother.[1,2] Common sense would virtually dictate that the status of the baby and mother is linked in an intimate and unique manner.

One of the more important categories of extrinsic insults is represented by microbial pathogens, which are the agents of intrauterine infection, as acquired through the maternofetal transplacental route or the ascending route, often through ruptured membranes.[3] The specific microorganisms and the time of the infection during gestation are two important variables that will determine the consequences to the embryo or fetus. Among viral infections, the effects on the unborn child are especially well known for rubella, cytomegalovirus and varicella-zoster.[4,5] Gregg made the pioneering observation that maternal rubella in the first trimester of pregnancy was associated with congenital cataracts.[6] Later, persistent patent ductus arteriosus and peripheral pulmonary stenoses were recognized as additional features of rubella embryopathy.[4] In the last 50 years, the list of pathogens has grown, and with it, an appreciation for the range of effects on the embryo and fetus. These range from teratogenicity, to inflammatory sequelae and intrauterine growth retardation.

This issue contains a study by Schwarz and colleagues, who have examined the organs of fetuses from mothers who had confirmed acute human parvovirus B19[B19] infections. These ultimately resulted in spontaneous termination of pregnancies in 16 of 19 cases, and one perinatal death.[7] A combination of old and new technologies (routinely stained microscopic sections and *in situ* DNA hybridization) established the presence of B19 in a number of organs, where the virus preferentially infects erythroblasts.[8–10] A comparison of the two techniques revealed somewhat greater sensitivity of *in situ* hybridization for the identification of infected cells, and the ability to detect the virus in autolyzed tissues that were unsuitable for histologic evaluation. An earlier study by Porter and associates examined lung tissues in 13 cases of unexplained hydrops fetalis, and found B19 DNA in blood vessels in four cases (31%).[12] They also identified characteristic pink to purple inclusions by light microscopy, but DNA hybridization demonstrated many more infected cells than were appreciated histologically. One obvious conclusion is that *in situ* hybridization improves sensitivity in addition to specificity for B19 infection.

It would seem that there is little room left for yet another virus that we must contend with after the human immunodeficiency virus, *Herpes simplex*, and human papillomavirus. However, the discovery of parvovirus B19 (B1 through B18 do not exist) in 1975 eventually established the etiology of "fifth disease" or erythema infectiosum, and explained aplastic crises in individuals with chronic hemolytic anemias.[11,13] Transient erythrocytic aplasia probably occurs in most patients with B19 infection. A self-limited symmetrical polyarthropathy also has been described in adults who lack evidence of a skin rash.

Several important questions emerge from the study of Schwarz and associates. One relates to the epidemiologic aspects of B19 infection, and the other to the morphologic manifestations in the fetus that would suggest the possibility of the infection. The above-cited study by Porter et al. addresses the latter question to some degree.[12] Schwarz and associates have shown that an acute B19 infection was confirmed in the mothers by the detection of viral specific IgM[7]; this antibody is found 3 to 4 days after the onset of maternal illness, and usually declines within 1 or 2 months. Nonetheless, questions remain. Were these mothers infected during a B19 epidemic, and were these cases collected during a period of time without well-defined epidemiologic implications? After all, it was during an epidemic of erythema infectiosum in Scotland that the first documented cases of B19-associated fetal hydrops and spontaneous abortion were identified.[14] On the other hand, Porter and co-workers selected their cases from examples of unexplained hydrops fetalis during a period of time without a known B19 epidemic.[12] Because it is accepted that B19 is transmitted vertically from the mother to the fetus, what is the probability of a fetal infection when the mother has an acute infection? There are rec-

ognized differences among the various types of viruses in infectivity rates and fetal effects, which are dependent to some extent on the time during gestation when the infection occurs. For instance, 1 to 2% of all newborns in the United States have serologic evidence of an *in utero* cytomegalovirus infection, but only 10% of neonates have overt clinical features of this infection.[5] Unlike the thorough documentation of maternofetal cytomegalovirus infection, comparable data are unavailable for B19. However, some inferences regarding risk have been derived from a few epidemiologic and anecdotal reports. It would appear that most fetuses are spared the adverse consequences of an acute maternal B19 infection. In a case-control study of congenital anomalies and fetal deaths (spontaneous abortions and stillbirth) Kinney and associates were unable to demonstrate excess fetal morbidity or mortality rates after an outbreak of B19 infection.[15] They also evaluated a group of mothers who experienced fetal wastage after a B19 epidemic and were unable to show B19 IgM in any patient or control subject. It was their conclusion that the rate of maternofetal B19 transmission was approximately 1%. Using several published and unpublished sources, the Committee on Infectious Diseases of the American Academy of Pediatrics concluded that the risk of presumed B19-related fetal death ranged from 3% to 9% in those mothers who were infected during the first 20 weeks of pregnancy.[16] A more ominous outcome was suggested by Rodis and colleagues, who reviewed the literature and examined their own experience. They concluded that 38% of pregnancies had an adverse outcome when the mother had an antenatal B19 infection.[17] All are in agreement on fetal risk, but there is no consensus about its level.

Hydrops fetalis is seemingly a consistent pathologic finding in the B19-infected fetus. With the marked decline in Rh incompatability-associated hydrops in most developed countries, nonimmune hydrops fetalis is encountered more commonly in a relative sense.[18] It is estimated that 1:1400 to 1:2500 pregnancies are complicated by the latter disorders. Its causes are multitudinous, ranging from hemoglobinopathies or cardiac (and other) anomalies, to infections. Approximately 5% of all cases of nonimmune hydrops fetalis are caused by a fetal viral infection (cytomegalovirus, rubella, herpes simplex, parvovirus), bacteria (syphilis), or protozoa (*Toxoplasma*).[19] The pathophysiology of parvovirus-associated hydrops fetalis is similar, in some respects, to the hemoglobinopathies and hemolytic anemias. These conditions feature a diminished volume of erthrocytes, because erthroblasts are destroyed as targets of the infection. Anemia, decreased plasma colloid osmotic pressure, and congestive heart failure, in concert, are the pathophysiologic factors in the develop-

ment of hydrops fetalis, it has an attendant mortality rate of more than 90%.[20] In addition to marked subcutaneous edema, effusions in major body cavities, inflammation of the heart and skeletal muscle, and abnormal hepatic extramedullary hematopoiesis, the spleen and kidneys are often abnormal in individual cases.[21] There also have been some reports suggesting that B19 may have teratogenic effects, with the occurrence of microphthalmia and retinal dysplasia, reminiscent of ocular rubella embryopathy.[22]

Parvovirus B19 has not acquired the widespread notoriety of rubella, *Herpes simplex*, or human immunodeficiency virus, as a harbinger of serious (if not lethal) consequences to the fetus and neonate. The pathologist should be alerted to the possibility of B19 infection if a thorough gross and microscopic examination of a stillborn hydropic fetus fails to yield an etiology. *In situ* hybridization is a powerful technique that can be used to confirm a B19 infection, but the starting point is the recognition of inclusion-bearing erythroid cells in the fetus and edematous placental villi.

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Original Article

Intraoperative Surgical Specimen Evaluation: Frozen Section Analysis, Cytologic Examination, or Both?

A Comparative Study of 206 Cases

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AND GEOFFREY MENDELSOHN, M.D.

Having recently become aware of the merits of cytologic preparations, histopathologists are focusing their attention on cytologic examination as a means of intraoperatively evaluating surgical specimens. This study compares the diagnostic accuracy and quality of frozen-section (FS) and cytologic preparations from 206 surgical specimens. The quality of cytologic preparations was significantly superior to that of FSs ($P = 0.0001$). With the use of a three-level accuracy scale suited to the practical demands of intraoperative evaluation, there was no significant difference between the accuracy of diagnosis by FS analysis compared with that achieved by cytologic examination ($P = 0.35$). More importantly—except in one case—whenever one technique did not correctly distinguish benign from malignant disease, the other technique yielded an essentially correct diagnosis. With the use of both techniques, 99.5% of cases were interpreted correctly, at least in regard to benign *versus* malignant diagnoses. Because significant additional time, equipment, stains, laboratory space, or personnel are not needed to implement intraoperative cytologic studies in a routine anatomic pathology laboratory, the authors advocate the simultaneous use of FS and cytologic studies in the specified context. (Key words: Intraoperative consultation; Intraoperative cytology; Frozen section; Fine-needle aspiration) Am J Clin Pathol 1991;96:8–14

Over the last 70–80 years, numerous studies throughout the world have found cytologic examination to be a rapid, accurate, and reliable means of diagnosing neoplastic and nonneoplastic processes. Having become aware of the many advantages offered by cytologic examination, when compared with those of frozen section (FS) analysis, histopathologists recently have refocused their attention on cytology as an adjunct to FS diagnosis in intraoperative surgical pathology. Studies comparing the diagnostic accuracy of FS analyses with that of intraoperative imprint and crush cytologic studies have recently appeared in the English literature. Most of these have concentrated on specific body sites such as the breast,[1,2] lymph nodes,[3,4] nervous system,[5,6] and salivary glands.[7,8] Although a few large studies, examining lesions from a variety of sites,

have been published since 1977,[9–11] most have considered only the distinction of benign from malignant disease. To our knowledge, none has objectively scored the degree of accuracy of diagnosis, nor has any study evaluated the quality of the specimens produced with each technique. Furthermore, no large assessment has used fine-needle aspiration (FNA), a cytologic technique that may reduce the risk of sampling errors.

To better assess the relative merits of intraoperative cytologic and FS studies, this article presents a series of 206 surgical specimens, each of which was processed simultaneously for cytologic studies and FS analysis. All preparations were evaluated for overall diagnostic accuracy and quality using point scoring systems.

MATERIALS AND METHODS

Specimens

The study comprises a representative sample of cases submitted to the Surgical Pathology Laboratory for intraoperative surgical pathologic evaluation during the 20-month period from August 1988 to March 1990. The

sample consisted of all cases received by one of us in which both intraoperative FS and cytologic studies were performed. Cases requiring microscopic assessment of surgical margins were excluded so that they could be considered in a separate study. Two hundred six cases were evaluated.

Frozen Sections

All tissue submitted for FS analysis was examined grossly. Areas that were most representative of the lesion in question were sectioned, embedded in OCT® compound (Tissue-Tek, Miles Inc., Elkhart, IN), frozen rapidly in a cryostat, and cut into 6-μm sections. These were fixed in absolute ethanol and stained with a rapid hematoxylin and eosin technique.

Cytologic Preparations

Whenever possible, FNA cytologic studies were undertaken on specimens that were received, which would enable maximal, thorough tissue sampling. FNA was performed using 22-gauge needles (outer diameter = 0.7 mm) according to the method described by Wilson and associates.[12] When specimens were too small or hard to aspirate (as in bony samples), scrape, crush, or imprint smears were prepared. All cytologic material was smeared evenly on glass slides, fixed in absolute ethanol, and stained in the same manner as FS slides.

Specimen Analysis

Analyses were performed retrospectively to ensure that the observers would have no recollection of final diagnoses in any particular case. All FS slides were assessed independently by two observers (assigned as FS-1 and FS-2). All cytologic slides were evaluated by a third observer. The three observers were provided with the clinical data available at the time of the initial surgical procedure, which included patient age, sex, site of the lesion, surgical gross description, and any additional relevant radiologic or clinical findings. All specimens were assessed for the quality of preparation and the degree of diagnostic accuracy.

Specimen Quality Assessment

A point score was assigned to each FS and cytologic preparation as follows:

0 points: The preparation exhibited such poor quality that the diagnosis was deferred necessarily (showing, for example, severe FS artifact, excessive tissue folding by FS, marked cellular degeneration, or inadequate volume of cellular material by cytologic examination).

1 point: The tissue sections or cytologic smears were so distorted that ease of diagnosis was compromised.

2 points: Cellular detail was preserved very well, with minimal distortion.

Diagnostic Accuracy Assessment

Each case was assigned one or more diagnoses. These were compared with the final pathologic diagnosis, based on permanent paraffin-embedded histologic sections, any special histochemical or immunohistochemical stains, and electron microscopic examination (when it had been performed). The following point scores were allocated to cases to denote degree of diagnostic accuracy:

0 points: The diagnosis was incorrect, with respect to benign *versus* malignant disease.

1 point: The diagnosis was correct regarding a benign *versus* malignant diagnosis only but incorrect with respect to the final diagnosis (*e.g.*, carcinoma *vs.* lymphoma);

  Or: The diagnosis was deferred until examination of permanent sections.

2 points: The diagnosis was "essentially correct," *i.e.*, correct regarding benign *versus* malignant status, and in terms of classification as carcinoma, sarcoma, inflammatory, *etc.*, but not "absolutely" correct (*e.g.*, duct instead of lobular carcinoma of breast; sarcoma not otherwise specified, instead of leiomyosarcoma);

  Or: The correct specific diagnosis was included in the differential diagnosis (*e.g.*, a case of adenocarcinoma called "adenocarcinoma *vs.* large cell undifferentiated carcinoma");

  Or: The diagnosis was correct and specific (*e.g.*, leiomyosarcoma, follicular non-Hodgkin's lymphoma, caseating granulomatous inflammation).

Each case was assigned one cytologic score for diagnostic accuracy and for quality and two scores for FS accuracy and quality. Each observer was scored by consensus for diagnostic accuracy. All accuracy points were allocated by considering the limitations of the analytic technique; for example, if a thyroid follicular carcinoma was identified as a "follicular neoplasm" by cytologic examination, two points were assigned because cytologic examination cannot differentiate reliably between follicular adenoma and carcinoma. Similarly, if a minimally invasive follicular carcinoma of the thyroid did not demonstrate capsular or vascular invasion on FS analysis, and the FS therefore was interpreted as "follicular adenoma, pending adequate sectioning of capsule," two points were allocated.

For the following reasons, some diagnoses were deferred until permanent sections were prepared (accuracy score = 1):

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1. The specimen was of poor quality, such that the quality score was 0 points;
2. Difficult diagnostic problems existed (*e.g.*, atypical reactive lymphoid hyperplasia *vs.* non-Hodgkin's lymphoma); and
3. There were limitations resulting from a small sample size.

Analysis of Results

Specimen quality and the diagnostic accuracy of each technique (cytologic and FS analyses) were evaluated to determine whether either method was superior in any way. Because FS analysis is the more accepted procedure, cytologic examination was tested under more stringent conditions, such that, in all cases, the points assigned to cytologic preparations were compared with the higher of the two scores for FSs (referred to as "better FS").

The cytologic scores for diagnostic accuracy and quality were compared with those for FS analysis in all 206 cases when considered cumulatively, as well as in regard to individual specific sites as considered independently. All values were interpreted statistically using (where appropriate) the sign test and the Wilcoxon unmatched-pairs signed-ranks test. A P value ≤ 0.05 was considered to denote statistical significance.

RESULTS

Sites

The 206 surgical specimens were obtained from a variety of body sites, as shown in Figure 1. Fifty-two (25.2%) were from the breast; 40 (19.4%) from lymph nodes; 21 (10.2%) from the central nervous system (14 cerebrum, 2 cerebellum, 4 meninges, 1 ulnar nerve); 18 (8.7%) from soft tissue; 16 (7.8%) from the female genital tract (2

FIG. 1. Sites of 206 study cases.

uterus, 13 ovary, 1 vagina); 15 (7.3%) from the respiratory tract; 14 (6.8%) from endocrine tissues (11 thyroid, 1 adrenal, 2 parathyroid); 10 (4.9%) from the gastrointestinal tract (2 anus, 2 colon, 4 small bowel, 2 stomach); 4 (1.9%) from liver; 4 (1.9%) from the urinary tract (2 bladder, 2 kidney); 3 (1.5%) from bone; 3 (1.5%) from the pancreaticobiliary system (2 periampullary, 1 gallbladder); 3 (1.5%) from skin; 2 (1.0%) from salivary gland (1 parotid and 1 submandibular); and 1 (0.5%) from the testis.

Cytologic Preparation

The 206 cytologic specimens included 110 (52.9%) fine-needle aspirates, 29 (13.9%) scrapes, 11 (5.3%) crush preparations, and 4 (1.9%) imprints. The remaining 52 (26.0%) were nonaspirated specimens for which the exact method of sampling was not further specified.

Scores for Accuracy of Diagnosis

The points scored for diagnostic accuracy with each technique are shown in Table 1 and Figure 2. By any single observer, fewer than 3% of cases were incorrect regarding classification as benign *versus* malignant. There was no significant difference in diagnostic accuracy between cytologic findings and the better FS score when all 206 cases were considered cumulatively ($P = 0.35$, Wilcoxon matched-pairs signed-ranks test) or any specific site that was examined was considered.

Among the total of 206 cases, there were 14 accuracy scores of 0 points from 11 cases, as shown in Table 2. Five of these 14 were obtained by cytologic examination, six by FS-1, and three by FS-2. Only one case was given a score of 0 by all three observers. In each of the other cases in which cytologic preparations were scored as 0, the better FS score was 2 points. Similarly, for each of the other instances in which FS was scored as 0, the cytologic specimen was scored as 2 points.

There were 19 instances in which the diagnostic accuracy score was 1 point; 5 of these results were obtained by cytologic examination, 5 by FS-1, and 9 by FS-2. The specifics of these cases are presented in Table 3.

In total, there were four true sampling errors in the study group; two were cytologic preparations and two cases were FS samples (see Table 2). Both cytologic specimens were scrape samples. FS was diagnostic in both, with the better FS score being 2 points in each. The cases involving FS true sampling errors both were diagnosed correctly by FNA cytologic examination.

Ten diagnoses were deferred until permanent histologic sections were prepared (see Table 3). Three of these con-
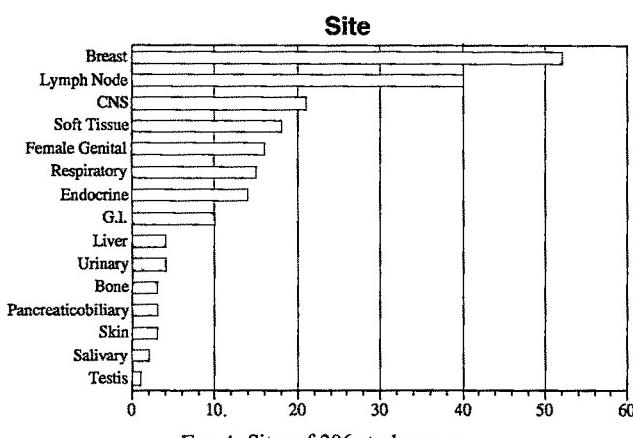
TABLE 1. DIAGNOSTIC ACCURACY SCORES
FOR EACH OBSERVER

Point Score (see text)	Cytology: No. of Cases (%)	FS-1: No. of Cases (%)	FS-2: No. of Cases (%)	Better FS: No. of Cases (%)
0	5 (2.4)	6 (2.9)	3 (1.5)	2 (1.0)
1	5 (2.4)	5 (2.4)	10 (4.8)	6 (2.9)
2	196 (95.2)	195 (94.7)	193 (93.7)	198 (96.1)
Totals	206 (100)	206 (100)	206 (100)	206 (100)

cerned cytologic specimens, compared with two by FS-1 and five by FS-2.

Specimen Quality Scores

The results of assessments of specimen quality are listed in Table 4. With the use of the sign test, cytologic examination was shown to be significantly superior to FS analysis (considering "better FS" values) when all 206 cases were considered cumulatively ($P = 0.0001$). When each specific site was evaluated, cytologic preparations of lymph nodes were of significantly better quality than FSs ($P = 0.013$, sign test). There were no significant differences in specimen quality in comparisons of the two techniques performed on specimens from any of the other specific sites, although the size of many of these site-specific groups was small.

DISCUSSION

Both FS and cytologic studies have been validated as acceptable techniques for the examination of intraoper-

Diagnostic Accuracy Scores
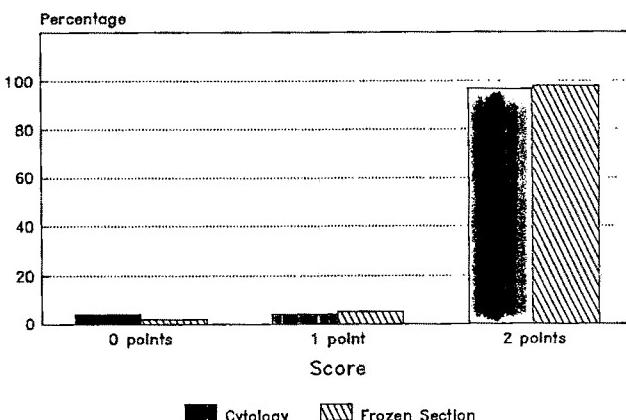
FIG. 2. Histogram showing number of cases scoring 0, 1, and 2 points for diagnostic accuracy by cytology and by frozen section (see text).

ative pathologic specimens. Each has its own merits and limitations. FS provides a display of tissue architecture that most closely approximates that of permanent histologic sections, enabling a degree of "comfort" for histopathologists in FS interpretation. In contrast, many observers are reluctant to assess cytologic smears, which do not reflect tissue architectural patterns as completely.

It is well recognized, however, that the freezing and sectioning techniques of FS result in unavoidable distortion and degeneration of tissue, rendering diagnosis difficult in many instances. Perhaps the greatest advantage of cytologic examination is the avoidance of such artifacts, resulting in superb nuclear and cytoplasmic detail. Minimal tissue is needed for cytologic examination; therefore, diagnosis of very small lesions is facilitated and tissue is

TABLE 2. CASES WITH A DIAGNOSTIC SCORE OF ZERO

Site	Final Diagnosis	Cytology Score	FS-1 Score	FS-2 Score
Breast	Infiltrating duct carcinoma	0*	0†	0†
Breast	Infiltrating duct carcinoma	0†	2	2
Chest wall	Invasive duct carcinoma	0†	2	2
Lymph node	HIV-associated lymphadenopathy	0*	2	1
Ovary	Corpus luteal cyst	0*	2	2
Arm	Malignant schwannoma	2	0*	2
Humerus	Organizing hematoma	2	0*	1
Lung	Carcinoid	2	0*	0*
Ampulla of vater	Adenocarcinoma	2	0†	0†
Thyroid	Minimally invasive follicular carcinoma	2	0*	2
Bone	Metastatic prostatic adenocarcinoma	2	2	0*

* Interpretative error.   † True sampling error.

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TABLE 3. CASES WITH A DIAGNOSTIC SCORE OF ONE

Site	Final Diagnosis	Cytology Score	F/S-1 Score	F/S-2 Score
Arm	Malignant schwannoma	1*	0	2
Breast	Colloid carcinoma	2	1	1
Breast	Fibrocystic disease	1	2	2
Breast	Radial scar	1	2	1*
Breast	Lobular carcinoma	1*	2	2
Lymph node	Non-Hodgkin's lymphoma	1*	2	2
Lymph node	HIV infection	0	2	1*
Lymph node	Reactive hyperplasia	2	1*	1*
Lymph node	Non-Hodgkin's lymphoma	2	1*	1*
Brain	Metastatic adenocarcinoma	2	1	1
Uterus	Papillary serous adenocarcinoma	2	1	2
Lung	Small cell undifferentiated carcinoma	2	2	1*
Retroperitoneum	Paraganglioma	2	2	1*
Humerus	Organizing hematoma	2	0	1

* Diagnosis deferred.

saved for permanent sections and special studies (such as flow cytometry, hormone receptor assays, and electron microscopic examination). Certain tissues that can be adequately assessed cytologically cannot be studied by FS for technical reasons (*e.g.*, bone, necrotic tissue, fat).

Because of the advantages offered by cytologic examination in intraoperative diagnosis, pathologists recently have refocused their attention on this technique as an adjunct to FS analysis. Articles comparing the two procedures have appeared in the English literature with increasing frequency since the late 1960s.[1,10] However, all of these are descriptive, rather than quantitative, statistical studies. All have shown cytologic examination to be useful in intraoperative evaluation of surgical specimens. No study has evaluated the degree of diagnostic accuracy that is possible with each technique or the quality of the specimens obtained with each of them. This study has addressed both of these issues by means of semiquantitative point-scoring systems.

Regarding specimen quality, cytologic examination yields significantly superior results ($P = 0.0001$) compared with FS analysis. In addition to crush, scrape, and imprint cytologic preparations, this study included cytologic evaluations obtained by FNA. We believe that FNA produced the best-quality cytologic preparations. Scrapes occasionally resulted in increased cellular trauma, making interpretation more difficult, especially in the evaluation of lymph nodes and benign breast lesions in which the scrape technique induced an artifactual loss of cellular cohesion.

In considering the degree of diagnostic accuracy, the data in this study show that the use of either FS or cytologic examination alone results in an acceptable rate of correct

diagnoses, at least with regard to interpretations of benign *versus* malignant (97.6%). However, when the two techniques were considered together, this diagnostic figure rose to 99.5% because there was only 1 of 206 cases (0.5%) in which a correct diagnosis (benign *vs.* malignant) could not be established.

Excluding the one case in which all three observers scored 0 points, FS analysis yielded an essentially or completely correct diagnosis (score 2 points) in all instances in which cytologic interpretations were incorrect or nonspecific (scores of 0 or 1). Similarly, excluding the same case, in all instances in which the FS diagnosis was incorrect or nonspecific, the cytologic findings were essentially or completely correct.

In considering all correct diagnoses (score 2), there were 16 cases in which the cytologic diagnoses were essentially correct when the diagnosis of the "better FS" was completely correct. Eight of these (50%) were cytologic diagnoses of mammary tissues in which an error occurred either between a diagnosis of fibroadenoma *versus* fibrocystic disease, or in the specific type of carcinoma that

TABLE 4. SCORES OF SPECIMEN QUALITY

Points (see text)	Cytology: No. of Cases (%)	F/S-1: No. of Cases (%)	F/S-2: No. of Cases (%)	Better F/S: No. of Cases (%)
0	1 (0.5)	4 (1.9)	1 (0.5)	1 (0.5)
1	28 (13.6)	114 (55.3)	80 (38.8)	65 (31.6)
2	177 (85)	88 (42.7)	125 (60.7)	140 (68.0)
Totals	206 (100)	206 (100)	206 (100)	206 (100)

was diagnosed in malignant lesions. In breast cytologic preparations, the findings of a small absolute tumor cell size and the presence of signet-ring cells (with or without eosinophilic intracytoplasmic inclusions) have been promoted as criteria suggesting lobular rather than ductal carcinoma. However, in samples from six proven ductal carcinomas in this study, both of these features were present in most cells that were examined. It is thus apparent that these features are not pathognomonic for lobular carcinoma of the breast.

Overall, ten diagnoses were deferred, three by cytologic examination and seven by FS analysis. Six of the ten (60%) were lymph nodes deferred for suspected lymphoma. Only one of these six was deferred on the basis of cytologic findings. Of the three cases deferred by cytologic results, one was handled in this way because of very poor cellularity (such that specimen quality was scored as 0 points). The other two cases were difficult diagnostic problems. All seven cases deferred on the basis of FS analysis were difficult diagnostic problems. In all cases in which diagnosis was deferred by any observer, an essentially correct interpretation still was obtained by one or the other technique.

In considering the four examples of true sampling errors, it is interesting to note that the two cytologic samples were both scrapes (*i.e.*, examinations of a single cut surface of tissues, as opposed to the more thorough examination of overall specimen volume that is achieved by FNA). Furthermore, both cases involving FS sampling errors were diagnosed correctly with the use of FNA cytologic examination. These findings suggest that FNA allows for superior diagnostic sensitivity by allowing maximal tissue sampling. This is expected to be especially true in cases of microscopic, grossly inapparent lesions. Additional studies addressing this issue must be performed to determine whether there is a significant difference in diagnostic accuracy between FNA and other cytologic methods.

In certain individual cases, either FS or cytologic analysis proved to be particularly advantageous in the establishment of a correct diagnosis. In one adenocarcinoma of the lung that was diagnosed by both FS analysis and FNA, numerous asbestos bodies were seen in the cytologic smears. None was seen on the FS or permanent hematoxylin and eosin–stained sections. The cytologic preparation prompted the ordering of iron stains, which confirmed the presence of ferruginous bodies in tissue sections.

During the course of case collection for this study, some specimens could not be prepared for FS. These included two ductal breast carcinomas (which were too small to allow for FSs as well as permanent sections, flow cytom-

etry, and hormone receptor analyses), one case of fat necrosis (which was too friable to section suitably), and two specimens of bone fragments (which could not be sectioned before decalcification). In an additional case, the specimen consisted of swabs from a septic site, and only smears could be performed. Cytologic examination alone was undertaken in all of these cases, and it yielded an essentially correct diagnosis in all of them. Cytologic smears in this study required an average of 2 minutes to prepare, compared with a 10-minute mean time for FS preparation. This proved advantageous, especially when numerous lymph nodes were received simultaneously for cancer staging procedures. The pathologist interpreted the cytologic specimen while waiting for the FS slides to be prepared; thus, neither procedure required additional professional time or increased overall turnaround time.

Frozen-section analysis was particularly helpful in one unexpected case of human immunodeficiency virus (HIV) infection in a lymph node. The numerous activated immunoblasts in this specimen were misinterpreted by cytologic examination as reflecting the presence of Hodgkin's lymphoma *versus* peripheral T-cell lymphoma. With the use of FS analysis, a benign reactive architectural pattern was apparent.

CONCLUSIONS

Using a semiquantitative point score system, this study compared the degree of diagnostic accuracy and specimen quality in FS and intraoperative cytologic preparations. Two hundred six surgical specimens were evaluated. Cytologic preparations were found to be significantly superior with respect to specimen quality ($P = 0.0001$). In considering diagnostic accuracy, there was no significant difference between the two techniques ($P = 0.35$). Each method alone allowed for a correct diagnosis (at least with regard to benign *vs.* malignant status) in at least 97.6% of cases. However, when both techniques were combined, this figure increased to almost 100% (205 of 206 cases). This result shows that cytologic and FS analysis are complementary rather than competitive techniques, which, when used as adjuncts to one another, offer the greatest opportunity for establishing a correct diagnosis. FS examination is the traditional mainstay of intraoperative surgical pathology. However, because cytologic examination neither adds significantly to the time needed for FS preparation nor imposes excessive additional demands on a routine anatomic pathology laboratory (in terms of equipment, stains, and personnel), we advocate the simultaneous use of FS and cytologic studies in the intraoperative evaluation of surgical specimens.

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Anaplastic Thyroid Carcinoma
Immunocytochemical Study of 32 Cases

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To study the histogenesis of and determine the most useful markers for diagnosing anaplastic thyroid carcinoma (ATC), 32 cases, including 2 with numerous osteoclast-like cells, were stained with a battery of antibodies to epithelial (keratin, epithelial membrane antigen [EMA], carcinoembryonic antigen [CEA]), mesenchymal (vimentin, desmin, muscle-specific actin [MSA], Factor VIII–related antigen [FVIII:RAg]), endocrine (thyroglobulin, calcitonin, chromogranin [Cg]), lymphocytic (leukocyte common antigen [LCA]), histiocytic (alpha-1-antitrypsin [α1AT], alpha-1-antichymotrypsin [α1AChy], KP1), melanocytic (HMB-45), and Schwann cell (S-100 protein) markers. Five tumors were associated with papillary carcinoma. In one of these cases, a morphologic continuum between the well-differentiated carcinoma and the ATC was visualized by their positive immunostaining for both vimentin and keratin, thus supporting the hypothesis that the latter tumor originated from the former. Twenty-five (78.1%) tumors expressed keratin, 10 (31.3%) reacted for EMA, and 3 (9.4%) expressed CEA, confirming the epithelial nature of this neoplasm. Reactivity for thyroglobulin was seen in a small number of cells in five (15.6%) ATCs. Because all of the cases that expressed keratin also stained positively for EMA, CEA, or thyroglobulin, it is believed that keratin is the most useful epithelial marker for diagnosis of ATC. A lack of reactivity for calcitonin and Cg indicates that these tumors are not derived from C cells, as has been proposed by some authors. Reactivity for KP1 (CD68), a monoclonal antibody that reacts with a macrophage-associated antigen, occurred in the osteoclast-like cells but not in the anaplastic tumor cells. This finding, together with negative keratin staining of the osteoclast-like cells, indicates that these cells are not epithelial in nature and therefore should be considered reactive rather than neoplastic. Thirty tumors (93.8%) expressed vimentin, 15 (46.9%) marked for α1AChy, 11 (34.4%) exhibited α1AT, and 11 (34.4%) expressed S-100 protein. Because all of these markers can be seen in a wide variety of tumors of different histogeneses, they have no value in the diagnosis of ATC. Although immunostaining for FVIII:RAg, desmin, and MSA was negative in all of these tumors, these markers can help to differentiate between ATCs and some soft tissue sarcomas with which they can be confused. (Key words: Anaplastic thyroid carcinoma; Thyroid gland; Immunocytochemistry; Osteoclast) Am J Clin Pathol 1991;96:15–24

The histogenesis of anaplastic thyroid carcinoma (ATC) has been a subject of controversy in the literature. Some studies,[1-3] particularly those from Europe, have indicated that many of these tumors represent thyroid sarcomas, whereas other studies[4-9] have demonstrated that they are indeed carcinomas. Some authors suggested that the large majority of ATCs originate from C cells and are, therefore, medullary carcinomas.[10,11] Currently, however, most pathologists agree that ATCs arise from follicular epithelial cells, probably by transformation of a preexisting well-differentiated carcinoma.[12-20]

Anaplastic thyroid carcinomas can present different histologic patterns. They may be composed of spindle cells arranged in interlacing fascicles or in storiform arrays; they also may be formed by poorly cohesive giant cells with abundant cytoplasm and eccentric nuclei or by solid groups of cells with squamoid features.[5,7,21] Because of this wide range of morphologic patterns, ATCs can be confused with various types of sarcomas, as well as with some other carcinomas, melanomas, and large cell lymphomas. Occasionally, large cell ATCs may contain numerous osteoclast-like giant cells. Whether these cells are neoplastic or reactive stromal cells also has been a subject of debate in the literature.[22-25] This article reports our experience with 32 cases of ATC, including 2 with numerous osteoclast-like cells, using a large panel of antibodies directed to different cell markers. These were selected to determine the best choice of markers to assist the pathologist in distinguishing such tumors from other poorly differentiated neoplasms with which they can be

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confused, to obtain a better understanding of the histopathogenesis of these tumors, and to elucidate the nature of the osteoclast-like cells in ATC.

MATERIALS AND METHODS

Thirty-two cases of ATC, in which sufficient material for immunohistochemical studies was available, were selected from the files of the Department of Pathology, The University of Texas M. D. Anderson Cancer Center. Hematoxylin and eosin–stained slides were reviewed in all cases, and the histologic patterns of the tumors were classified as follows, according to a modified version of the criteria of Carcangiu and associates[5]: (1) spindle cell—tumors composed predominantly of spindle cells resembling fibrosarcoma or malignant fibrous histiocytoma; (2) giant cell—tumors made up of large pleomorphic cells often lining bizarre, sometimes multiple, hyperchromatic nuclei with abundant eosinophilic cytoplasm; (3) squamoid—tumors with areas resembling nonkeratinizing squamous carcinoma; and (4) mixed—tumors with two or more of the previous patterns.

Immunocytochemical studies were performed on formalin-fixed, paraffin-embedded tissue sections with the use of the avidin–biotin–peroxidase complex (ABC) method of Hsu and associates.[26] The specimens were cut at 3–4 μm thickness, deparaffinized in xylene, and rehydrated in descending grades of ethanol (100–70%). To enhance the immunostaining with some antibodies, the sections were digested with 0.1% protease (type XIV; Sigma Chemical Company, St. Louis, MO) in phosphate-buffered saline, pH 7.6, for 30 minutes; for the remaining antibodies, this step was omitted because the enzymatic treatment produced a detrimental effect on final immunostaining results. The use of digestion is specified according to antibody in Table 1.

Endogenous peroxidase activity was blocked by a 10-minute treatment with 3% hydrogen peroxide in absolute methanol. Sections then were incubated in a humid chamber with the primary antibodies (Table 1) for one hour at room temperature. This procedure was followed by immunoperoxidase staining with the use of ABC Elite® kits (Vector Laboratories, Burlingame, CA). To minimize background staining, preincubation with normal horse

TABLE 1. PRIMARY ANTIBODIES, THEIR SOURCES, DILUTIONS, AND ENZYMATIC TREAMENTS

Antibody	Source	Dilution	Enzymatic Digestion
Antivimentin MoAb	DAKO Corporation Santa Barbara, CA	1:25	No
Anti-CEA MoAb	Hybritech Inc. San Diego, CA	1:1,000	No
Anti-EMA MoAb	DAKO Corporation	1:20	Yes
AE1/AE3 keratin MoAb[13] (broad range of type I and type II keratins)	Boehringer Mannheim Indianapolis, IN	1:300	Yes
CAM 5.2 keratin MoAb[27] (39, 43, 50 kDa)	Becton Dickinson Mountain View, CA	1:2	Yes
34βH11 keratin MoAb[28,29] (45, 52 kDa)	Enzo Biochem New York, NY	1:2,000	Yes
35βE12 keratin MoAb[28,29] (57, 66 kDa)	Enzo Biochem	1:2,000	Yes
Rabbit antithyroglobulin	Ortho Diagnostics Raritan, NJ	1:3	No
Antithyroglobulin MoAb	Medica Carlsbad, CA	1:10	No
MSA (HHF35 MoAb)[30]	Enzo Biochem	1:800	No
Rabbit anticalcitonin	Immunonuclear Stillwater, MN	1:2,000	No
Rabbit anti-FVIII:RAg	DAKO Corporation	1:200	Yes
Antidesmin MoAb	DAKO Corporation	1:10	Yes
Rabbit anti-S-100 protein	DAKO Corporation	1:700	No
Rabbit anti-α1AT	DAKO Corporation	1:4,000	Yes
Rabbit anti-α1AChy	DAKO Corporation	1:2,000	Yes
Chromogranin A (KL2H10 MAb)[31]	Boehringer Mannheim	1:40	No
KP1 MoAb (CD68)[32]	DAKO Corporation	1:500	Yes
HMB-45 MoAb[33]	Enzo Biochem	1:750	Yes
Leukocyte common antigen	DAKO Corporation	1:40	Yes

MoAb = monoclonal antibody; CEA = carcinoembryonic antigen; EMA = epithelial membrane antigen; MSA = muscle specific actin; FVIII:RAg = Factor VIII-related antigen; α1AT = alpha-1-antitrypsin; α1AChy = alpha-1-antichymotrypsin.

serum was used for monoclonal antibodies and normal goat serum was employed for polyclonal antibodies (1:10 dilution). Immunostains were developed with the use of 3-amino-9-ethylcarbazole as chromogen. The slides were counterstained with Mayer's hematoxylin. To evaluate the specificity of the antibodies, known positive and negative tissues were used as controls. Details of the immunostaining procedure have been described elsewhere.[34]

RESULTS

Light Microscopy

Of the 32 cases, 18 showed a single histologic pattern (8 giant cell, 6 squamoid, and 4 spindle). Thirteen of the remaining cases were composed of a mixture of spindle and giant cells, whereas the last case had squamoid areas in addition to spindle and giant cells. Two of the tumors that contained a large population of multinucleated osteoclast-like giant cells were included in the mixed group because the anaplastic cells presented spindle and large cell morphology. Five cases (three giant cell and two mixed spindle and giant cell) were associated with a papillary carcinoma of the thyroid.

Immunocytochemistry

Table 2 summarizes the immunostaining results for the different antibodies used in this study. Twenty-seven (84.3%) of the 32 tumors reacted for keratin. The largest

number of these positive cases (25 cases) were labeled with the use of a "cocktail" of AE1/AE3 monoclonal antibodies that recognizes a wide range of high and low molecular weight keratins. This was followed by CAM 5.2 (23 cases) and 34βH11, (22 cases) two monoclonal antibodies that react with low molecular weight (acidic) keratin polypeptides. Only 11 cases stained with the 35βE12 antibody, which recognizes high molecular weight (basic) keratins. Although all tumors with a squamoid pattern reacted with this antibody, it appeared to be much less sensitive in staining neoplasms with other histologic patterns (Fig. 1). In contrast, other antibodies did not appear to show a marked preference for tumors with any particular histologic pattern.

Thirty tumors expressed vimentin, and 25 (75.3%) coexpressed vimentin and keratin. In most cases, this coexpression occurred not only in the same tumors, but also in the same cells. The staining was distributed evenly throughout the cytoplasm, but in three tumors it assumed the form of paranuclear globoid inclusions. One of the tumors, which was associated with a papillary carcinoma of the thyroid, reacted for vimentin and keratin in a similar staining pattern as that seen in the anaplastic carcinoma (Figs. 2 and 3).

Only ten tumors reacted for epithelial membrane antigen (EMA). The staining in these cases was strongest

TABLE 2. IMMUNOCYTOCHEMICAL RESULTS FOR 32 CASES OF ANAPLASTIC THYROID CARCINOMA

| Antibody | Histologic Pattern | | | | |
	Giant Cell (n = 8)	Spindle (n = 4)	Squamoid (n = 6)	Mixed (n = 13)	Total
AE1/AE3 antikeratin	5	3	6	11	25 (78.1%)
34βH11 antikeratin	6	2	6	10	24 (75%)
CAM 5.2 antikeratin	5	2	6	10	32 (71.9%)
35βE12 antikeratin	0	1	6	4	11 (34.4%)
Antivimentin	8	4	5	13	30 (93.8%)
Anti-α1AT	3	2	1	5	11 (34.4%)
Anti-α1AChy	3	3	2	7	15 (46.9%)
Anti-CEA	0	0	2	1	3 (9.4%)
Anti-EMA	0	0	5	5	10 (31.3%)
Antithyroglobulin (MoAb)	3	1	0	1	5 (15.6%)
Antithyroglobulin (PAb)	3	1	0	0	4 (12.5%)
Anti-S-100	3	2	1	5	11 (34.4%)

α1AT = alpha-1-antitrypsin; α1AChy = alpha-1-antichymotrypsin; CEA = carcinoembryonic antigen; EMA = epithelial membrane antigen; MoAb = monoclonal antibody; PAb = polyclonal antibody.

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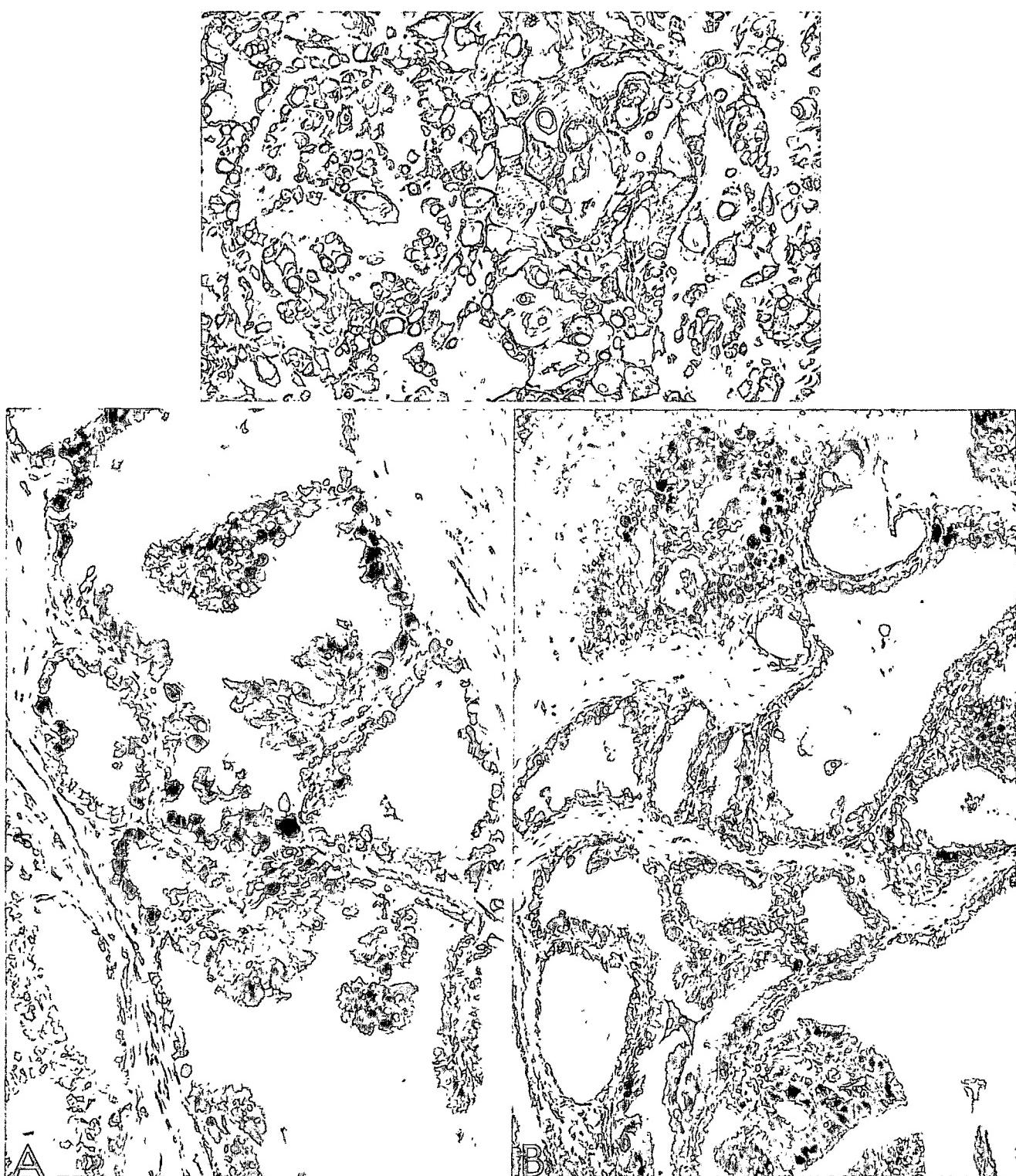
FIG. 1 (*upper*). Anaplastic carcinoma stained with 35βE12 antikeratin antibody showing positive reactivity in squamoid areas of the tumor. Avidin-biotin-peroxidase complex (×300).

FIG. 2 (*lower*). *A.* Papillary carcinoma of the thyroid showing paranuclear reactivity for vimentin. *B.* Anaplastic carcinoma area reacting for vimentin (top). Portion of the papillary carcinoma is seen at the bottom of this figure. Avidin-biotin-peroxidase complex: *A,* (×100); *B,* (×150).
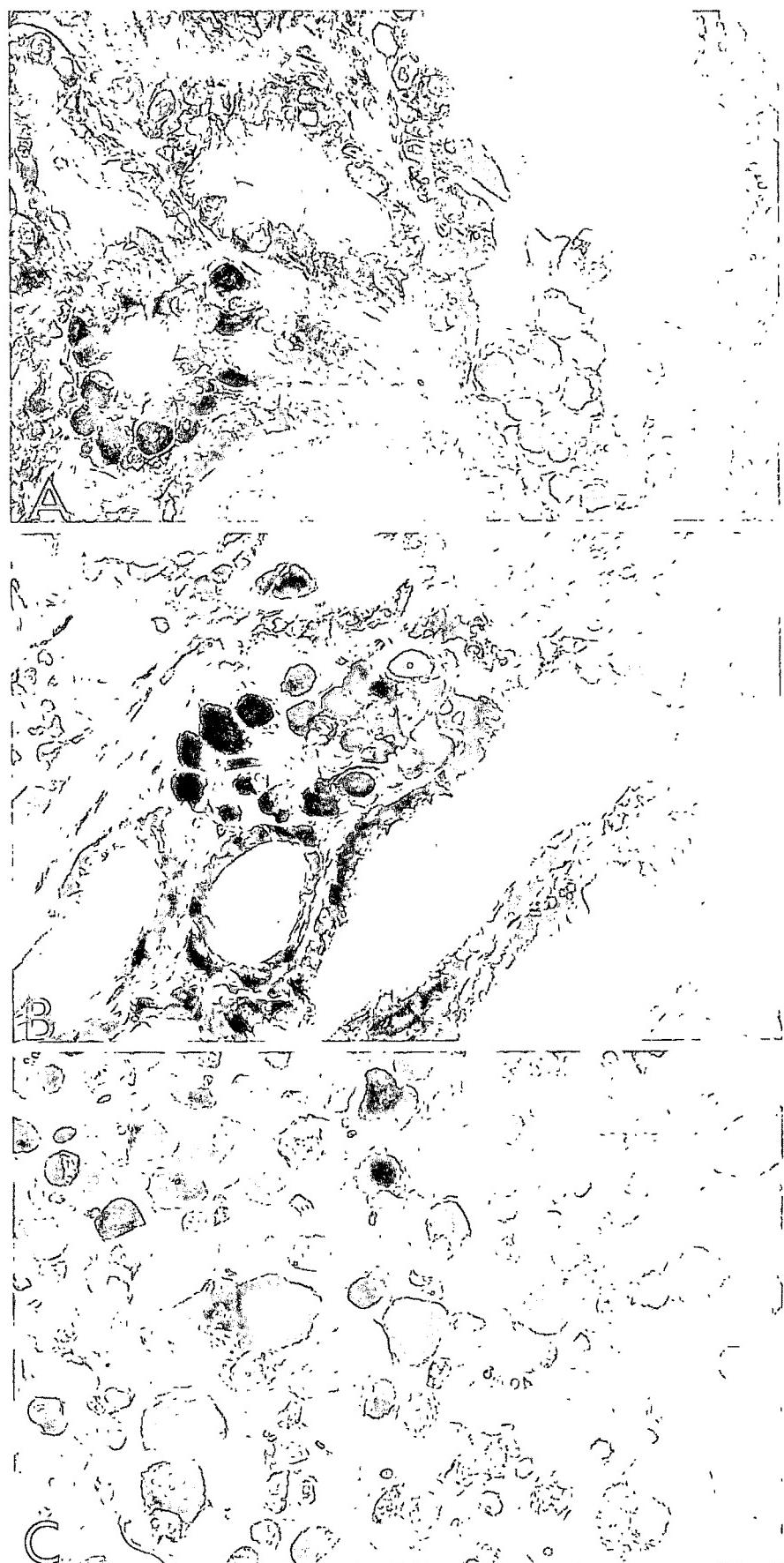
FIG. 3. Same case as shown in Figure 2 stained for vimentin. *A.* Early papillary proliferation showing paranuclear reactivity. *B.* Small clusters of follicular cells (*upper right*) having similar paranuclear staining pattern as that of the small anaplastic carcinoma (*center*). *C.* Area of poorly cohesive anaplastic carcinoma cells displaying numerous paranuclear globoid-like inclusions reacting for vimentin. Avidin-biotin-peroxidase complex: *A, B,* and *C* (×250).

along the cell membrane (Fig. 4). All tumors that were labeled for EMA also expressed keratin. The intensity of the staining was stronger for keratin than for EMA. Three tumors (two squamoid and one mixed) reacted for carcinoembryonic antigen (CEA).

Five tumors expressed thyroglobulin in 1–5% of the tumor cells. Four of these lesions reacted with both of the antithyroglobulin antibodies used in this study. Serial sections in such cases did not disclose the presence of entrapped normal thyroid cells or well-differentiated thyroid carcinoma.

Eleven tumors stained positively for S-100 protein. Reactivity was observed in the cytoplasm and nuclei of the cells. In two of these cases, S-100 was expressed in more than 80% of the cells, whereas staining was focal in the remaining cases.

Eleven cases reacted for alpha-1-antitrypsin (α1AT), and 15 expressed alpha-1-antichymotrypsin (α1AChy). No preference was noticed for a particular type of histologic pattern with either of these markers.

The KP1 monoclonal antibody produced strong and uniformly positive staining of the multinucleated osteoclast-like cells, whereas it only focally stained mononuclear and binucleated cells with bland cytologic features; it did not react with anaplastic spindle or giant cells, normal thyroid epithelium, or papillary carcinoma cells (Fig. 5). A few osteoclast-like cells also were labeled weakly for vimentin, α1AT, and α1AChy but did not stain for S-100 protein, keratin, EMA, or CEA. None of the tumors expressed calcitonin, chromogranin A, desmin, muscle-specific actin (MSA), Factor VIII-related antigen (FVIII: RAg), or leukocyte common antigen (LCA), nor did they stain with the HMB-45 monoclonal antibody.

DISCUSSION

Five (15.6%) of the 32 anaplastic tumors were associated with papillary carcinoma of the thyroid. This incidence, although somewhat low, falls within the range (8.5[35] to 80%[36]) that has been reported in the literature regarding the association of anaplastic carcinoma with a well-differentiated form of thyroid cancer. The fact that a significant number of anaplastic carcinomas are associated with well-differentiated tumors has led some authors to postulate that ATC originates from a well-differentiated thyroid carcinoma.[12,14–18,20] The demonstration in our study of a continuous spectrum of morphologic changes and the likeness in the distribution of intermediate filaments in anaplastic carcinoma with those seen in the papillary tumor strongly support this hypothesis.

The results of this study indicate that—from a practical point of view—keratin is the most useful marker for diagnosing anaplastic carcinoma. We demonstrated reactivity for this marker in 25 (78.1%) of the 32 tumors. These results agree with those of other investigators who have demonstrated keratin expression in 40–100% of cases.[5,6,9,37] The wide range of results between series could result from differences in fixation procedures, use of proteolytic enzymatic treatment of the specimens to enhance keratin immunostaining, or differences in the spectrum of reactivity of antibodies that recognize different keratin polypeptides. We could not demonstrate reactivity for keratin in seven cases, despite prolonged proteolytic digestion of the tissues. Histologically, these tumors were similar to those staining for keratin. Although we cannot be certain as to what caused the negative immunostaining results, the observation that four cases showed entrapped follicular cells that reacted only weakly for this marker indicates that the negative results could be attributed, at least in part, to overfixation of the tissues. It is known that formalin reduces the immunoreactivity of keratin and that prolonged fixation may result in negative immunostaining for this cytoskeletal protein.

Although one cannot exclude the possibility that some of these cases may represent another type of anaplastic tumor, the fact that none of the tumors reacted for LCA, HMB-45, FVIII:RAg, desmin, or MSA and the knowledge that they presented clinically as rapidly growing thyroid tumors are indications that these cases were, indeed, carcinomas. Sarcomas of the thyroid are extremely rare, with the possible exception of angiosarcomas, which appear to occur endemically in some Alpine areas.[7,38]

In this study, we found that antibodies that react with low molecular weight keratins stained the largest number of tumors, regardless of their histologic pattern. Antibodies directed to high molecular weight keratins were less sensitive and had a tendency to react more frequently with tumors displaying a squamoid pattern. Keratin and vimentin coexpression was not surprising because it has been reported in normal thyroid epithelial cells.[37] In this series, we demonstrated coexpression of these intermediate filaments in approximately two-thirds of the cases; such results are comparable to those reported by others (17.5–100%).[4,6,39–41] Coexpression of keratin and vimentin may occur in a wide variety of carcinomas originating in different sites, especially tumors with sarcomatoid features.[42,43]

Only a relatively small number of tumors reacted for EMA and CEA. The fact that all tumors that stained positively for these markers also expressed keratin is an indication that both markers have rather limited value in the diagnosis of ATC. Furthermore, because some large cell lymphomas may express EMA,[44,45] we believe that positive immunostaining for this marker should be interpreted with caution in those cases in which the possibility of an anaplastic large cell lymphoma is considered in the
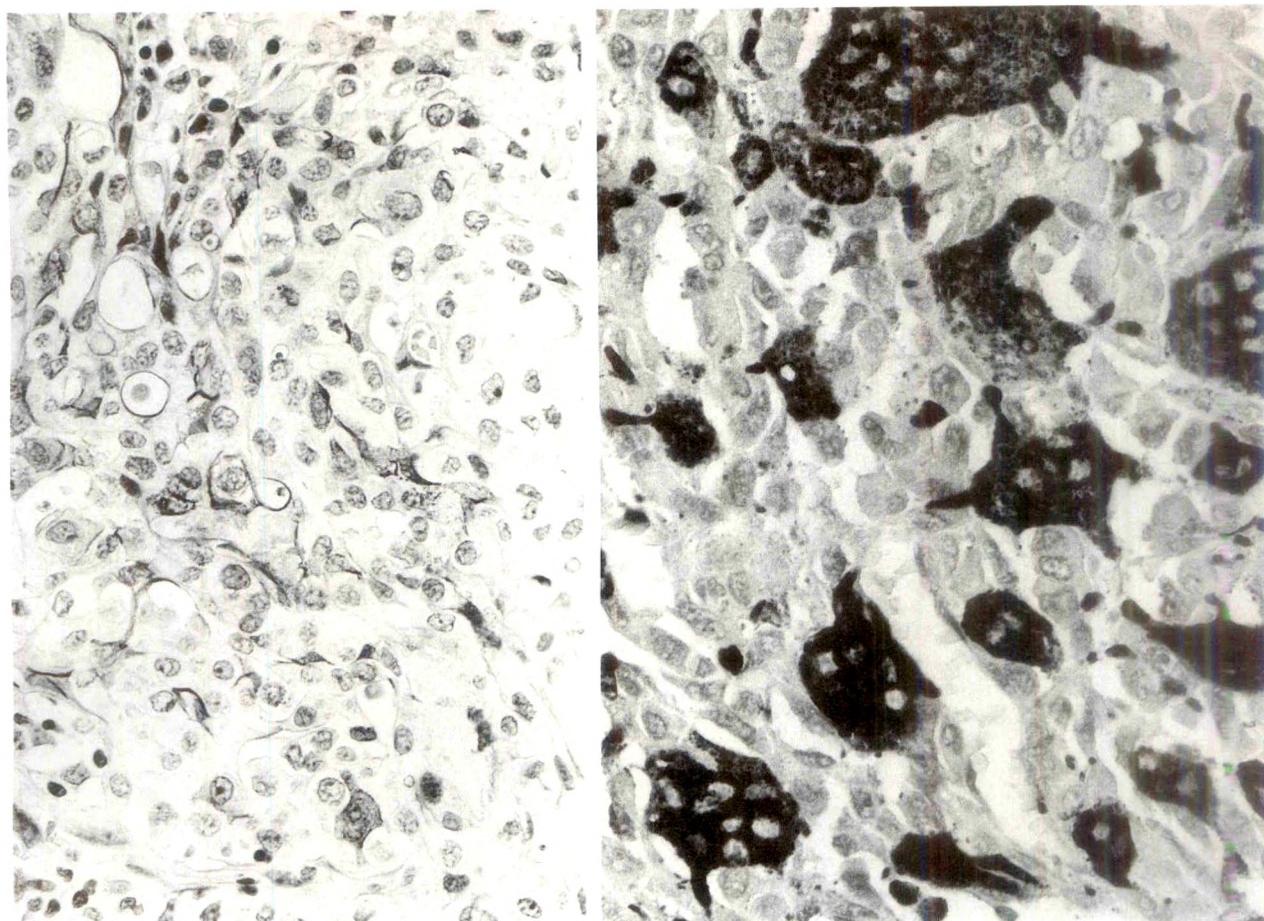
FIG. 4 (*left*). Anaplastic carcinoma showing peripheral staining pattern for epithelial membrane antigen. Avidin-biotin-peroxidase complex (×300).

FIG. 5 (*right*). Tumor staining preparation showing numerous osteoclast-like and sparse mononuclear cells reacting with KP1 monoclonal antibody. Avidin-biotin-peroxidase complex (×300).

differential diagnosis. In this context, immunostaining for keratin and LCA may aid in confirming the epithelial nature of the tumor.

In contrast to keratin, the value of thyroglobulin in the diagnosis of ATC has been a source of continuous controversy. Some studies reported that as many as 70% of ATCs express this marker,[4,8,39,46–49] whereas others have been unable to find thyroglobulin reactivity in any cases.[5,43] The cause of these discrepancies is not completely clear. In a recent study, de Micco and associates[47] indicated that the type of antibody used was the main source of such disagreement. They obtained reactivity for thyroglobulin in 17% of anaplastic carcinomas stained with a polyclonal antibody and with one monoclonal antibody, whereas 50–70% of the same cases reacted with another three antithyroglobulin monoclonal antibodies. Only a small number of tumors in the current series reacted for thyroglobulin. The staining in all of these cases was weak and limited to a few tumor cells. Therefore, our results

appear to support those studies indicating that thyroglobulin is a poor immunocytochemical marker for anaplastic carcinoma, at least with the antibodies we employed.[5,6,39]

Some series[10,11] indicated that most anaplastic carcinomas express calcitonin and are, therefore, a variant of medullary carcinoma. However, the uniform negativity of our cases for calcitonin and chromogranin fails to support a C-cell origin for anaplastic carcinomas. We should mention that medullary carcinoma of the thyroid presenting an anaplastic large cell pattern may indeed occur, although, in our experience, this occurrence is extremely rare. Among 200 cases of medullary thyroid carcinoma studied at this institution, we have seen only one example of such a tumor (unpublished observation).

Large numbers of osteoclast-like cells characterized two of the tumors in this series; but this type of pattern is uncommon. In 1983, Esmaili and associates[50] found only 21 such cases in the literature. The nature of the osteoclast-like giant cells has been debated for some time. Some

authors[7,22,51] consider them to be benign reactive cells of histiocytic origin; however, others[23,24,52] believe that the cells have an epithelial origin and are, therefore, malignant because they also can be found in blood vessels near the tumor and in metastatic sites.[53]

The observation that the osteoclast-like multinucleated giant cells in our cases reacted with the KP1 monoclonal antibody, but not with any of the antikeratin antibodies, is conclusive evidence against an epithelial origin for these cells. KP1 is a recently developed monoclonal antibody directed against a lysosomal fraction of human lung macrophages that recognizes a fixative-resistant antigen present in a wide variety of tissue macrophages[32] (designated antigen CD68 by the Fourth Workshop on Human Leukocyte Differentiation Antigens held in Vienna in 1989[54]). In contrast to other "histiocytic" markers, such as α1AT and α1AChy, KP1 is highly specific for histiocytic cells. The finding that a number of mononuclear and binucleated cells with bland histologic features also reacted with this antibody suggests that the multinucleated giant cells may have been formed by a coalescence of such cells.

Some authors[55,56] have indicated that α1AT and α1AChy are two "histiocytic" markers that can be used to differentiate malignant fibrous histiocytoma (MFH) from other neoplasms. Our finding that these markers were expressed in a relatively large number of tumors, regardless of their histopathologic patterns, confirms the results of other studies that indicate these markers are not specific for histiocytes. Because of their distribution in a wide variety of carcinomas, sarcomas, lymphomas, and melanomas, they have limited practical use in the diagnosis of neoplastic conditions.[57,58]

The demonstration of S-100 protein in roughly one-third of the cases was interesting. This observation is important because this marker often is used to support a diagnosis of melanoma, which can exhibit a histologic pattern that is similar to that of ATC. None of these cases stained with HMB-45, a monoclonal antibody that reacts with the large majority of melanomas. In our experience,[59] as well as that of others,[33] HMB-45 is extremely helpful in the diagnosis of melanoma.

None of the tumors in this series reacted for any muscle markers (desmin and MSA). These results indicate that antibodies against desmin and MSA can help distinguish anaplastic carcinomas from leiomyosarcomas, rhabdomyosarcomas, and MFHs; all of which are tumors with which anaplastic carcinoma may be confused. In our experience, as well as that of others,[60,62] leiomyosarcomas and rhabdomyosarcomas usually react strongly for desmin and with the HHF35 monoclonal antibody, which recognizes cardiac, smooth, and skeletal muscle actins. In addition, because MSA and desmin can be expressed fo-

cally in a number of MFHs,[61,63] immunostaining for these markers could be helpful in differentiating some MFHs from ATCs.

In conclusion, immunocytochemistry represents an extremely helpful ancillary method in the histopathologic diagnosis of ATC. Based on the results of this study, keratin is the most useful tissue marker for the recognition of this tumor, whereas, in our experience, CEA and EMA have only limited diagnostic value. We could not confirm the results of previous studies indicating the utility of thyroglobulin in the diagnosis of ATC. The finding of a morphologic continuum between well-differentiated carcinoma of the thyroid and anaplastic carcinoma, visualized by immunostaining for vimentin and keratin supports the hypothesis that the latter tumor originates by anaplastic transformation (clonal evolution) of the former. Finally, the demonstration of a histiocyte/macrophage phenotype in osteoclast-like giant cells that are present in some anaplastic tumors provides proof that these cells are not neoplastic in nature.

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The Human Hematopoietic Progenitor Cell Antigen (CD34) in Vascular Neoplasia

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The human hematopoietic progenitor cell antigen CD34 is synthesized and expressed by early normal hematopoietic progenitor cells and by many acute leukemias. Anti-CD34 antibodies also have been reported to stain blood vessels in tissue sections, and, more recently, CD34 mRNA has been detected in vascular endothelial cells. Therefore, the authors studied the diagnostic utility of immunohistochemical CD34 antigen detection in tumors of endothelial cell derivation and compared the results with stains for von Willebrand (vW) factor. A wide variety of epithelial and mesenchymal neoplasms also were examined to assess the specificity of CD34 for vascular neoplasia. Seven cases of angiosarcoma (seven of seven), five cases of Kaposi's sarcoma (five of five), and eight cases of epithelioid hemangioendothelioma (eight of eight) were moderately to strongly positive for CD34. This reactivity was equally intense in frozen sections, alcohol-fixed tissue, and formalin-fixed specimens. In many cases, the malignant endothelial cells stained more strongly than adjacent benign endothelium. Moreover, in most cases CD34 positivity was quantitatively and qualitatively stronger than staining for vW factor. Two cases of hemangiopericytoma (two of two) were CD34 positive but stained less intensely than the angiosarcomas, Kaposi's sarcomas, or hemangioendotheliomas. Five of six cases of hemangioma also stained positively for CD34; the nonreactive tumor in this group was the only one among 28 vascular neoplasms studied that was not reactive for CD34. In comparison, 9 of the 28 vascular tumors did not stain for vW factor. Three hundred fifty-seven tumors of nonvascular derivation also were examined for CD34 antigen expression. Focal light staining was seen in one pulmonary squamous cell carcinoma; moderate to intense staining was observed in half of the epithelioid sarcomas studied (8 of 16) and in a minority of leiomyosarcomas (3 of 22). These findings indicate that CD34 is a sensitive and relatively specific marker for neoplasms of vascular origin. (Key words: CD34; Angiosarcoma; Kaposi's sarcoma; Vascular neoplasia) Am J Clin Pathol 1991;96:25-31

The CD34 antigen is an approximately 115-kD cell surface protein that is expressed selectively by human hematopoietic (myeloid and lymphoid) progenitor cells.[1-3] To date, the diagnostic utility of CD34 antibodies primarily has involved the recognition of acute leukemias. Approximately 30–60% of acute leukemias are positive for CD34, and the expression of this antigen may have adverse prognostic significance in this setting.[4-6] The biochemical structure and partial amino acid sequence of the CD34 molecule have been described in detail,[7] and the CD34 gene has been mapped to chromosome 1q.[8] However, the exact role of the CD34 antigen in early hematopoiesis remains unclear.

In addition to its presence in early human hematopoietic cells, several studies have also demonstrated CD34 antigen positivity in vascular endothelium.[9,10] More recently, expression of the CD34 gene in vascular endothelial cells has been documented.[11] In other analyses, cultured human umbilical vein endothelium preferentially expressed CD34 in a small subset of cells with the morphologic appearance of migrating cells.[12] These findings suggest that CD34 may be expressed selectively in endothelial cells during angiogenesis.

To test the utility of CD34 antigen expression as a diagnostic marker for vascular neoplasia, we have studied a variety of endothelial cell–derived tumors with a monoclonal antibody to CD34 and compared these results with staining for the Factor VIII–related antigen, now known as von Willebrand (vW) factor. In addition, many tumors of nonvascular derivation also were studied for CD34 reactivity.

Original Article

MATERIALS AND METHODS

Twenty-eight vascular tumors of various types were studied, as outlined in Table 1. Most cases were derived from the files of the Sylvia Cowan Surgical Pathology Laboratory and from the consultation files of one of the authors (H.B.). Dr. Jerome Taxy, of Lutheran General Hospital, Park Ridge, Illinois, contributed four cases. The tissues were fixed in neutral buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin for routine histologic examination. In nine cases, B5-fixed, alcohol-fixed, or frozen tissue also was available for study. We reviewed all cases, and the original diagnosis was confirmed with standard morphologic criteria. Supportive ultrastructural studies were performed previously in seven cases.

With the use of single-tumor and multitumor tissue blocks,[13] a variety of neoplasms of nonvascular origin also were examined for CD34 immunoreactivity, as outlined in Table 2.

Immunohistochemical Studies

In all cases, immunohistochemical studies were performed on either formalin-fixed or B5-fixed paraffin-embedded tissue sections. A modification of the avidin–biotin complex technique was used to identify CD34 and vW factor, as described previously.[14] Trypsin digestion (0.1% for 30 minutes) preceded vW factor antibody staining; no digestion was done before CD34 staining. Primary antibody incubations were performed overnight at 4 °C.

Frozen tissue sections also were studied in five cases, and ethanol-fixed tissue sections were evaluated in two cases. For these studies, the period of primary antibody incubation with anti-CD34 was 30 minutes.

Antibodies

The antihuman progenitor cell antigen antibody (My-10; CD34) from Becton Dickinson (Mountain View, CA) was used at a dilution of 1/30. Monoclonal anti-vW factor antibody was purchased from DAKO (Santa Barbara, CA) and used at a dilution of 1/20.

RESULTS

The results of the immunohistochemical detection of the CD34 antigen in 28 cases of vascular neoplasia are summarized in Table 1. CD34 antigen positivity was detected in all cases except for one hemangioma. In general, the staining intensity of the neoplastic vascular endothelium was greater than that of adjacent normal endothelial cells in the same tissue sections. Furthermore, except in

TABLE 1. CD34 AND vW FACTOR REACTIVITY IN VASCULAR TUMORS

			Staining Intensity	
Case	Tumor	Site	CD34	vW Factor
1	Angiosarcoma	Soft tissue, thigh	++	−
2	Angiosarcoma	Skin, hand	+++	−
3	Angiosarcoma	Breast	++	+
4	Angiosarcoma	Breast	+++	+
5	Angiosarcoma	Skin, leg	++	+
6	Angiosarcoma	Skin, arm	++	+++
7	Angiosarcoma	Skin, arm	++	+
8	Kaposi's sarcoma	Lymph node	+++	++
9	Kaposi's sarcoma	Stomach	+++	+
10	Kaposi's sarcoma	Lymph node	+++	+
11	Kaposi's sarcoma	Chest wall	++	+++
12	Kaposi's sarcoma	Lung	+++	+
13	Epithelioid hemangioendothelioma	Soft tissue, knee	+++	−
14	Epithelioid hemangioendothelioma	Soft tissue, neck	++	−
15	Epithelioid hemangioendothelioma	Lymph node	++	+
16	Epithelioid hemangioendothelioma	Bone	++	++
17	Epitheloid hemangioendothelioma	Liver	+++	+++
18	Epitheloid hemangioendothelioma	Breast	+++	−
19	Epitheloid hemangioendothelioma	Soft tissue, forearm	++	+
20	Epitheloid hemangioendothelioma	Abdomen	+++	−
21	Hemangiopericytoma	Meninges	+	+++
22	Hemangiopericytoma	Soft tissue, back	+	+
23	Hemangioma	Skin	+++	+++
24	Hemangioma	Liver	+	−
25	Hemangioma	Soft tissue, back	+	+++
26	Hemangioma	Spleen	+	++
27	Hemangioma	Skin	−	++
28	Hemangioma	Intramuscular	++	+++

+++ = Intense positive staining in the majority of neoplastic cells; ++ = light to moderate staining in the majority of neoplastic cells; + = light staining in a minority of neoplastic cells; − = no staining of neoplastic cells.

In all cases, CD34 and vW Factor staining was seen in non-neoplastic endothelial cells.

**TABLE 2. THE CD34 ANTIGEN IN TUMORS
OF NON-VASCULAR ORIGIN**

Diagnosis	Number of Positive Cases/Number of Cases Studied
Epithelial tumors	
Lung cancer	
Adenocarcinoma	0/13
Oat cell carcinoma	0/10
Squamous cell carcinoma	1/10
Undifferentiated	0/6
Liver cancer	
Hepatoma	0/28
Cholangiocarcinoma	0/14
Metastasis	0/16
Breast duct carcinoma	0/49
Colonic adenocarcinoma	0/24
Thyroid papillary carcinoma	0/2
Prostate carcinoma	0/5
Renal cell carcinoma	0/3
Ovarian carcinoma	0/2
Pancreatic carcinoma	0/2
Gastric carcinoma	0/2
Mesothelioma	0/3
Hodgkin's and non-Hodgkin's lymphoma	0/20
Melanoma and clear cell sarcoma	0/33
Mesenchymal tumors	
Epithelioid sarcoma	8/16
Leiomyosarcoma	3/22
Synovial sarcoma	0/17
Rhabdomyosarcoma	0/10
Ewings sarcoma	0/3
Alveolar soft part sarcoma	0/2
Malignant fibrous histiocytoma	0/5
Liposarcoma	0/3
Fibrosarcoma	0/3
Undifferentiated spindle cell sarcoma	0/26
Osteogenic sarcoma	0/1
Schwannoma	0/4
Malignant schwannoma	0/2
Chondrosarcoma	0/1
Total	12/357

In all tumor samples, non-neoplastic endothelial cells were immuno-reactive with CD34.

hemangiomas, staining of CD34 was often more intense and more widespread than that of vW factor.

All seven cases of angiosarcoma were strongly positive for CD34 (Fig. 1). Frozen tissue sections, ethanol-fixed tissue sections, and formalin-fixed specimens from one case displayed similar staining intensity. Benign endothelial cells also stained positively for both CD34 and vW factor in all seven tumors. The neoplastic cells were labeled for vW factor in five of these cases; two of the angiosarcomas did not stain for this antigen.

All five Kaposi's sarcomas showed strong CD34 antigen reactivity. In two cases in which frozen tissue was available, the formalin-fixed tissue sections and the frozen tissue sections were equally positive. The one case in which B5-fixed tissue was available also was stained strongly, with no appreciable increase in background staining (Fig.

2). As was seen in the angiosarcomas, staining of the malignant cells in Kaposi's sarcoma was often stronger than that seen in adjacent benign endothelial cells. The neoplastic cells in all five cases also stained positively for vW factor. However, in four of the five tumors, CD34 staining was distinctly stronger than vW factor reactivity.

All eight epithelioid hemangioendotheliomas showed moderate to strong CD34 antigen positivity. Staining of intracytoplasmic lumina in the malignant cells was especially evident (Fig. 3). Equally strong positive staining was seen in formalin-fixed and B5-fixed tissue in one case. Only four of the eight tumors was labeled for vW factor.

Two hemangiopericytomas were examined, and the neoplastic cells in both cases stained positively for CD34. However, in contrast to the strong positivity seen in angiosarcomas, Kaposi's sarcomas, and hemangioendotheliomas, the staining intensity of the neoplastic cells in the hemangiopericytomas was weak. vW factor staining was also slight in one of these cases and negative in the other.

CD34 staining in benign vascular neoplasms was variable. One of the hemangiomas examined was negative for CD34; this was the only CD34-nonreactive vascular tumor in the series. vW factor staining of the hemangiomas was also variable, but was stronger than that of CD34 in four cases.

To test the specificity of CD34 for tumors of vascular origin, 357 neoplasms of nonvascular derivation were stained for this antigen (Table 2). Within the group of epithelial tumors, focal light CD34 positivity was seen in only one lung squamous cell carcinoma. Tumors of melanocytic origin and a variety of lymphomas were uniformly negative. One hundred fifteen tumors of mesenchymal derivation were studied; unexpectedly, 8 of 16 epithelioid sarcomas in this group were reactive with My-10. In addition, a minority of leiomyosarcomas (3 of 22) were also positive; the staining intensity in these 3 cases was intense. All other tumors showed no CD34 antigen staining.

DISCUSSION

Anti-CD34 antibodies detect a 115-kD membrane antigen found specifically on human hematopoietic progenitor cells.[1-3] Although the CD34 antigen is detectable in only 1–4% of normal marrow cells, CD34-positive cells isolated from the bone marrow account for more than 90% of the hematopoietic cells capable of forming *in vitro* colonies.[2,3] Approximately 30–60% of acute leukemias, of lymphoid and myeloid origin, are CD34 positive,[2,5,6,10] but chronic leukemias and lymphomas have not expressed this antigen.[2] CD34 antigen positivity is associated with an immature blast cell phenotype in acute myelogenous leukemia and may be an adverse prognostic indicator in

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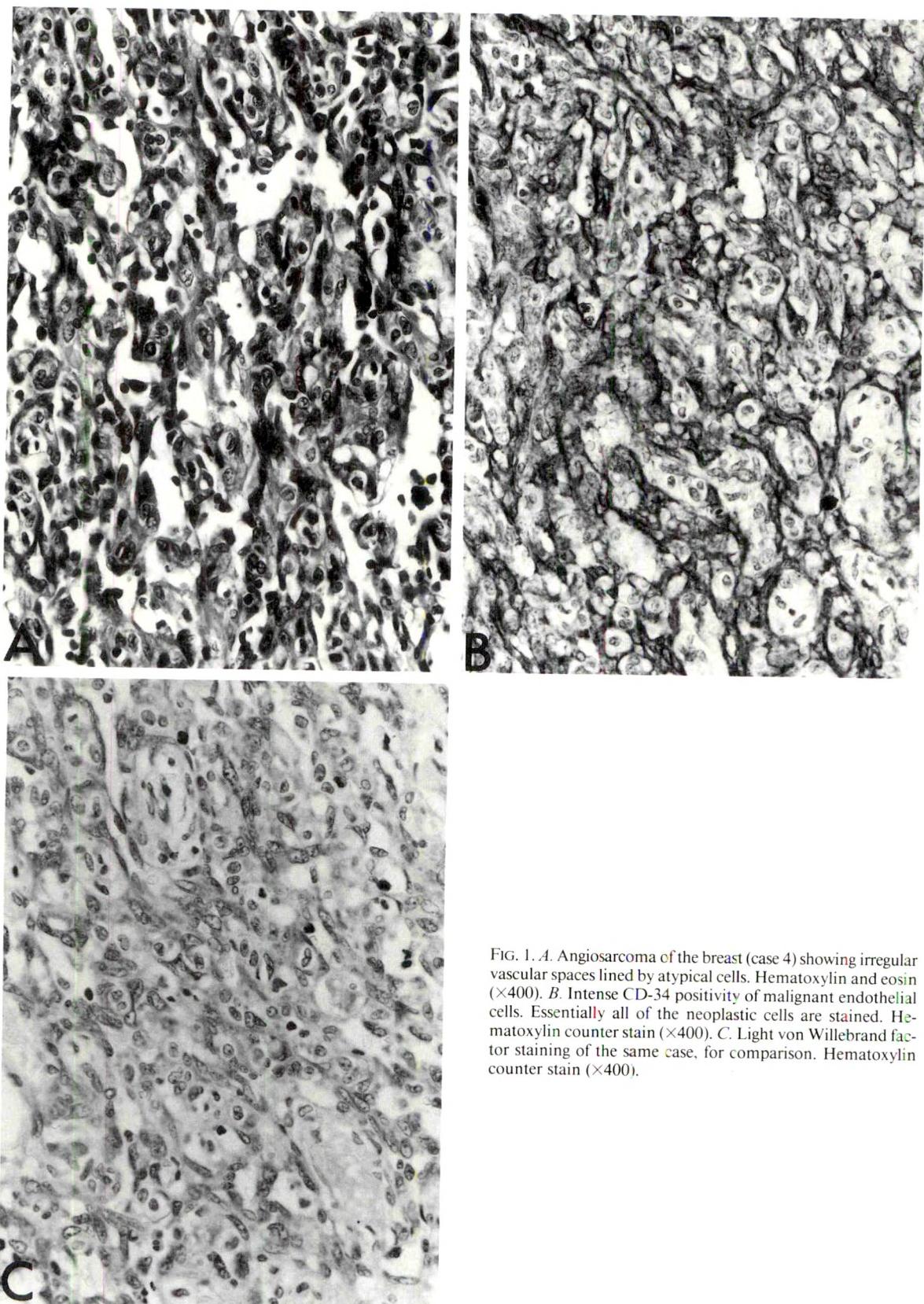
FIG. 1. *A.* Angiosarcoma of the breast (case 4) showing irregular vascular spaces lined by atypical cells. Hematoxylin and eosin (×400). *B.* Intense CD-34 positivity of malignant endothelial cells. Essentially all of the neoplastic cells are stained. Hematoxylin counter stain (×400). *C.* Light von Willebrand factor staining of the same case, for comparison. Hematoxylin counter stain (×400).
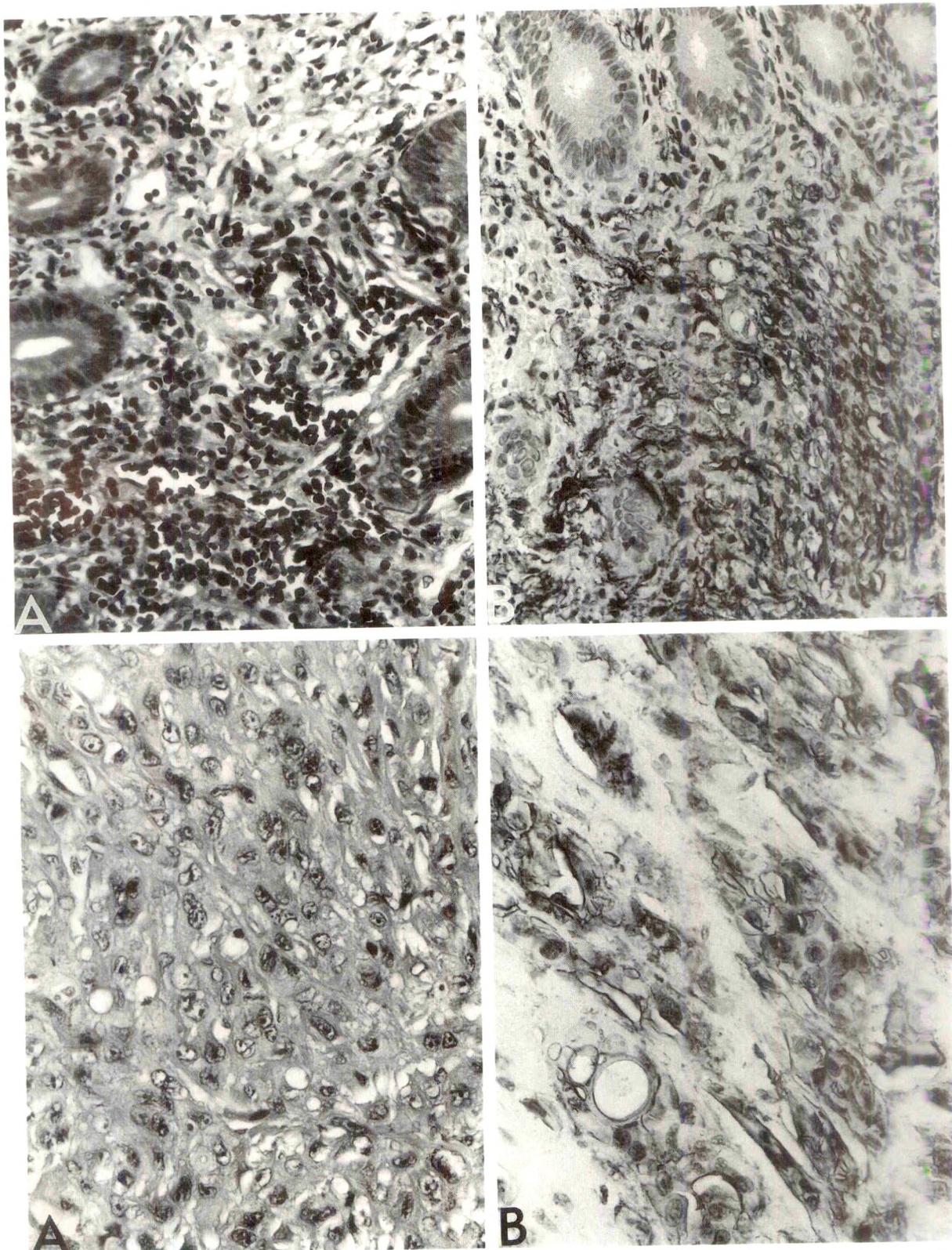
FIG. 2 (*upper*). *A.* Gastric Kaposi's sarcoma (case 9) fixed in B-5. Hematoxylin and eosin (×400). *B.* Strong positive staining for CD-34 is present in the vast majority of tumor cells. Hematoxylin counter stain (×400).

FIG. 3 (*lower*). *A.* Lymph node epithelioid hemangioendothelioma (case 15) showing characteristic features of abundant eosinophilic cytoplasm and intracellular lumina. Hematoxylin and eosin (×400). *B.* Strong CD-34 staining is present in the majority of neoplastic cells. The pattern of positive staining accentuates the cytoplasmic lumina. Hematoxylin counter stain (×400).

this setting.[4-6] Several previous studies also have noted CD34 antigen positivity in endothelial cells.[9,10] More recently, CD34 mRNA was found in cultured umbilical vein endothelial cells, indicating that the binding of CD34 antibodies to vascular endothelium is to the actual CD34 gene product, and not to a cross-reactive epitope.[11]

A cDNA clone of the CD34 gene, which has been localized to chromosome 1q,[8] recently was isolated and sequenced.[11] The predicted amino acid sequence deduced from that of the cDNA sequence suggests that the CD34 antigen is a typical membrane protein with a hydrophobic transmembrane region, separating a cytoplasmic carboxyl tail from the extracellular portion of the molecule.[7,11] The general structural features of the CD34 glycoprotein, its relatively high abundance on the cell surface, and its association with regions of cell contact in endothelial cells suggest that its function may be related to cellular adhesion, migration, or both.

Because benign endothelial cells have been shown to be reactive for the CD34 antigen, we tested a variety of vascular neoplasms for this protein. The most significant finding of this study is that the vast majority of vascular neoplasms are positive for CD34. Furthermore, the antigenicity of the CD34 protein is preserved in a wide variety of fixatives. Staining was equally intense, whether the tissue was fresh-frozen or fixed in ethanol, formalin, or B5.

All cases of angiosarcoma, Kaposi's sarcoma, and epithelioid hemangioendothelioma were strongly positive for CD34. The positive staining primarily was localized to the cell membranes and was especially conspicuous in cytoplasmic lumina. As is often found in immunohistochemical studies, less differentiated tumors tended to stain somewhat less intensely than the better differentiated ones. vW factor staining ranged from absent to strong, but in general was distinctly less intense than that of CD34. In addition, CD34 staining of neoplastic endothelial cells was often stronger than that of adjacent benign capillary endothelium. This finding suggests that the CD34 protein may be expressed at higher levels in neoplastic cells than in nonneoplastic cells, and it is consistent with the notion that the protein may function during endothelial cell proliferation (*i.e.*, during angiogenesis).[11,12]

Two hemangiopericytomas also stained positively for CD34, but the staining intensity was much less than that seen in angiosarcomas, Kaposi's sarcomas, or hemangioendotheliomas. This finding is somewhat at variance with the results of Schlingemann and colleagues, who found no CD34 expression in normal pericytes or perivascular fibroblasts.[12] However, the presence of low-level CD34 expression in malignant pericytes is not unreasonable because endothelial cells and pericytes may be derived

from a common precursor.[15] Furthermore, weak vW factor positivity, which was also present in one of our cases, has been reported previously in hemangiopericytoma.[16]

Five of six hemangiomas expressed CD34, but the staining of these benign vascular neoplasms varied and, in general, was not intense. In fact, the only vascular neoplasm not reacting with anti-CD34 belonged to this group. Because the function of the CD34 glycoprotein may be connected with endothelial cell proliferation,[11,12] the weak CD34 reactivity seen in hemangiomas could result from limited expression of the protein in these slowly proliferating neoplasms. The poor expression of CD34 in these benign tumors does not detract greatly from its diagnostic utility, however, because hemangiomas usually are easy to recognize on routine sections and rarely require special studies for diagnosis.

Our study of a large number of nonvascular tumors indicates that the CD34 antigen, as detected with the My-10 antibody, is not entirely specific for vascular endothelium. However, in this series, CD34 antigen expression in nonvascular tumors was limited to epithelioid sarcoma, with positive findings in half of the cases (8 of 16), and rare cases of leiomyosarcoma. In addition, one case of squamous cell carcinoma displayed slight and focal positive staining for CD34. The biologic significance of CD34 positivity in a substantial number of epithelioid sarcomas is unclear. However, in practical terms, epithelioid sarcoma is relatively unlikely to be confused morphologically with a vascular tumor. Furthermore, strong keratin expression is the rule in epithelioid sarcoma[17] and is only exceptionally present in vascular tumors.[18,19] The use of an appropriate immunohistochemical panel should readily distinguish between the two tumor types.

Although we found no positive CD34 staining in mesenchymal tumors other than epithelioid sarcoma and rare leiomyosarcomas, Ramani and colleagues recently reported 17 of 45 spindle cell tumors (primarily dermatofibrosarcoma protuberans and neural neoplasms) to be reactive with a CD34 antibody (QBEND/10) that was produced by injecting mice with endothelial cell membranes.[20] However, Watt and associates have demonstrated previously that all CD34 antibodies are not equal and that differences in antibody reactivity can be attributed to the recognition of distinct epitopes by different CD34 reagents.[10] A difference in epitope recognition sites may explain the apparent staining differences seen in the current series and in the report of Ramani and colleagues.[20]

Our study of vascular tumors for vW factor expression parallels the experience of others. The results are often disappointing because of the weak and sparse staining in a number of cases.[21-23] In general, the less differentiated

the vascular neoplasm, the more unlikely it is for one to observe vW factor staining. *Ulex europaeus* is not a marker that necessarily improves the recognition of vascular neoplasia. Although *U. europaeus* probably is more sensitive than vW factor in the detection of vascular lesions,[24,25] it is not endothelial cell specific, because binding of this lectin to carcinomas of both transitional and glandular origin has been described previously.[25]

In conclusion, our studies indicate that the CD34 antigen is a sensitive marker for vascular endothelium, both benign and neoplastic. The finding that malignant endothelial cells stain more intensely than their benign counterparts supports the notion that the CD34 protein may function in endothelial cell proliferation or migration. However, CD34 is not a specific marker of endothelial cells. In addition to normal hematopoietic precursor cells and acute leukemia cells, we found that two other solid tumor types that are not of vascular derivation also may express the antigen. Despite its less restricted specificity for endothelial cells, CD34 is still superior to vW factor in the detection of vascular neoplasms. The use of an appropriate antibody panel, chosen after narrowing the differential diagnosis by conventional means, increases the utility of CD34 as an endothelial cell marker.

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Comparative Immunohistochemical Staining for Desmin and Muscle-Specific Actin

A Study of 576 Cases

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Muscle-specific actin (MSA) and desmin are considered to be sensitive and specific markers for muscle differentiation. The authors compared staining patterns for these markers in 576 samples of normal, reactive, and neoplastic tissues. The standard avidin–biotin–peroxidase complex technique was performed with the use of two commercial antibodies against MSA (HHF35; Enzo Biochemical, Inc., New York, NY) and desmin (DER11; DAKO Corporation, Santa Barbara, CA), respectively, on consecutive paraffin-embedded tissue sections from these cases. Both MSA and desmin were found in all 80 normal muscle samples. Although MSA appeared diffusely in all vascular smooth muscle samples, desmin was demonstrated focally in vascular smooth muscle cells in 100 of 196 samples. MSA but not desmin always was found in myoepithelial cells (25 samples), pericytes (286 samples), and decidual cells (7 samples). Among 76 cases of myofibroblast-containing lesions, 14 and 54 were found to have desmin and MSA, respectively. MSA and desmin were found in 4 of 4 cardiac rhabdomyomas, 34 of 34 rhabdomyosarcomas, and 5 of 6 leiomyomas. Among 22 leiomyosarcomas, 7 displayed either MSA or desmin and 7 showed both markers. In general, more tumor cells showed staining for MSA than desmin, but the reverse was true in some cases. Tissue fixed in Zenker's solution seemed to show a significant decrease in MSA immunoreactivity, but no significant change for desmin staining was observed. None of the 154 normal tissues and 22 benign nonmyogenic tumors expressed MSA or desmin. Among 133 malignant nonmyogenic tumors, positive staining for both desmin and MSA was found in 3 of 8 cases of glioblastoma multiforme, 1 of 10 malignant schwannomas, and 1 of 14 malignant fibrous histiocytomas; staining for only MSA was found in 3 of 14 malignant fibrous histiocytomas, 1 of 10 malignant schwannomas, 6 of 6 fibromatoses, 1 of 1 mammary myofibroblastoma, and 1 of 7 malignant mesotheliomas; and staining for desmin only was seen in 1 of 7 malignant mesotheliomas. The authors conclude the following: (1) MSA is found consistently in muscle cells, pericytes, decidual cells, and myoepithelial cells, and frequently in myofibroblasts; (2) although the type of fixative does not influence the staining for desmin significantly, Zenker's fixative causes a marked decrease in the intensity of MSA staining; (3) both MSA and desmin are sensitive and specific markers for rhabdomyosarcoma; (4) for the diagnosis of formalin-fixed leiomyosarcomas, the sensitivities of MSA and desmin are roughly equal, but both are rather low; simultaneous staining for both antigens significantly increases the diagnostic yield but will not detect every case of leiomyosarcoma, especially those in the gastrointestinal tract; and (5) as many as 17% of nonmyogenic sarcomas display focal but unequivocal staining for MSA, desmin, or both. (Key words: Tumors; Immunohistochemistry; Desmin; Muscle-specific actin) Am J Clin Pathol 1991;96:32–45

It can be difficult to diagnose myogenic sarcomas histologically because they sometimes share remarkably similar morphologic features with many other types of tumors not derived from muscle.[1,2] Over the last decade, developments in immunohistochemistry significantly have enhanced the pathologist's ability to distinguish myogenic from nonmyogenic tumors. Panels of antibodies directed against various components of normal myocytes have been tested for their diagnostic utility. They include polyclonal or monoclonal antibodies directed against actin, creatine kinase, desmin, myoglobin, myosin, titin, tropomyosin, and Z-protein.[3–29] Almost without exception, initial enthusiasm was dampened subsequently by data indicating that the antibodies in question detected only a small number of myogenic tumors (low sensitivity) or also stained nonmyogenic tumors (low specificity).[8,11–14,16,19,20,24,25,29–31] Until recently, data from several systematic studies, including our own,[32] have suggested

that desmin is the most sensitive and specific marker for muscle differentiation.[4,33-36] On the other hand, several antibodies against actin, probably because of their polyclonal nature, display significant cross-reactivity to cells other than myocytes.[8,37-43]

In 1987, the monoclonal antibody HHF35, which is directed against muscle-specific actin (MSA), was developed and commercialized[44,45]; it has been considered to be a better marker for muscle differentiation than desmin.[46-51] Although several studies have used the HHF35 antibody as a marker for muscle differentiation,[45-51] only one has been devoted to a systematic evaluation of this antibody.[47] This article systematically compares the diagnostic utility of desmin and MSA and reviews the experiences of other researchers concerning the HHF35 antibody.

MATERIALS AND METHODS

Tissue Samples

Paraffin-embedded tissue samples used in this study are listed in Tables 1–7. The samples included normal muscle (skeletal, cardiac, vascular, and nonvascular smooth muscle) and various normal tissues other than muscle (Tables 1 and 2). Cardiac rhabdomyomas, uterine and gastric leiomyomas, and other benign spindle cell tumors also were examined (Table 4). In addition to rhabdomyosarcoma (RMS) and leiomyosarcoma (LMS), a wide variety of malignant neoplasms that might simulate LMS and RMS histologically were studied (Tables 5 and 6). Normal, reactive, and neoplastic tissues known to contain myoepithelial cells, or myofibroblasts, also were included (Table 3). Forty-two benign and malignant epithelial tumors were studied (Table 7).

Rhabdomyosarcoma or LMS was diagnosed if a tumor exhibited the generally accepted clinical and histologic characteristics of these tumor types[1] and if one or more of the following features were documented: desmin positivity, MSA positivity, and muscle differentiation by electron microscopic examination.[52-56] Electron microscopic studies were done in 23 RMSs and 14 LMSs, respectively.

TABLE 1. NORMAL MUSCLES

	Number of Cases	Positive Cases	
		Desmin	Actin
Skeletal muscle	31	31	31
Cardiac muscle	7	7	7
Smooth muscle, vascular	196	100	196
Smooth muscle, nonvascular	42	42	42
Total	276	180	276

TABLE 2. NORMAL TISSUES OTHER THAN MUSCLE

	Number of Cases	Positive Cases	
		Desmin	Actin
Chondrocyte	1	0	0
Chorionic villous stromal cell	9	0	2
Decidual cell	7	0	7
Endometrial stromal cell	9	0	0
Hematopoietic cell	3	0	0
Hepatocyte	6	0	0
Lymphoid cell	11	0	0
Neuron and glial cell	7	0	0
Osteocyte	8	0	0
Ovarian stromal cell	5	0	0
Epithelia	104	0	0
Total	170	0	9

Antigenic preservation was considered adequate when there was positive staining of built-in controls, such as normal muscle, by either antidesmin or anti-MSA; positive MSA staining for pericytes or vascular smooth muscle cells; or positive staining of mesenchymal cells for vimentin.[46]

Five hundred seventy-six cases (excluding cases of pericytes and vascular smooth muscle cells, which were used as built-in controls) were evaluated. However, more than one type of tissue might be present on tissue sections, and each was considered a "case" and tabulated accordingly.

Tissue Processing

Most materials were fixed in 10% buffered formalin for 3–34 hours. Several tumors obtained before 1982 were fixed in Zenker's solution; this group included 18 RMSs, 2 cardiac rhabdomyomas, 6 neuroblastomas, 4 Ewing's sarcomas, and 1 rhabdoid tumor. Six osteosarcomas and three chondrosarcomas included in this study required decalcification with 50% formic acid and 50% formalin for one to three days. Nevertheless, these tumors exhibited adequate antigenic preservation by one of the above criteria.

To evaluate the influence of fixatives on the staining outcome, adjacent tissue samples from normal muscle (three samples of myometrium, one of appendix, and one of detrusor muscle) were fixed separately in different fixatives, including 10% buffered formalin, absolute ethanol, and Zenker's, Bouin's, and B5 fixatives for six hours; then they were color-coded and embedded in the same block for comparison. To evaluate the influence of decalcification on staining quality, after four hours of fixation in formalin, adjacent samples of duodenal wall were decalcified (50% formalin and 50% formic acid) for a period of 1, 2, 4, and 18 hours; colored-coded; and embedded together in the same block for comparison. Mercury pig-

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TABLE 3. MYOEPITHELIAL CELLS AND MYOFIBROBLASTS

	Number of Cases	Positive Cases	
		Desmin	Actin
Myoepithelia			
Eccrine sweat gland	14	0	14
Salivary gland	6	0	6
Mammary gland	4	0	4
Bronchial gland	1	0	1
Subtotal	25	0	25
Myoepithelium-containing tumors			
Benign mixed tumor	3	0	0
Malignant mixed tumor	1	0	0
Subtotal	4	0	0
Myofibroblast in			
Atheromatous plaque	3	0	3
Tumor-associated desmoplasia	49	11	39
Granulation tissue	5	2	3
Keloid	5	0	1
Subtotal	62	13	46
Myofibroblast-containing tumors			
Fibromatosis	6	0	6
Benign fibrous histiocytoma	7	1	1
Myofibroblastoma	1	0	1
Total	105	14	79

ment was removed effectively from Zenker-fixed tissue as follows: tissue was placed in Weigert's iodine (2 g potassium iodide + 1 g iodine crystals + 100 mL distilled water) for 5 minutes, washed once in distilled water, placed in 5% sodium thiosulfate for 1 minute, and washed in running tap water for 5 minutes.

Immunostaining

The standard avidin–biotin–peroxidase complex technique was employed, with minor modifications. The details of this technique have been described elsewhere.[32,57] Primary antibodies included monoclonal anti-MSA (HHF35, 1:1,280 dilution; Enzo Biochemical, Inc., New York, NY) and antidesmin (DER-11, 1:50 dilution; DAKO Corporation, Santa Barbara, CA). Our previous

TABLE 4. BENIGN TUMORS

	Number of Cases	Desmin Only	Actin Only	Both	Neither
Myogenic tumors					
Leiomyoma	4	1	1	4	0
Cardiac rhabdomyoma	4	0	0	4	0
Subtotal	10	1	1	8	0
Non-myogenic tumors					
Meningioma	4	0	0	0	4
Neurofibroma	4	0	0	0	4
Schwannoma	3	0	0	0	3
Hemangioma	2	0	0	0	2
Cardiac myxoma	1	0	0	0	1
Subtotal	14	0	0	0	14

TABLE 5. RHABDOMYOSARCOMA AND SIMULATORS

	Number of Cases	Desmin Only	Actin Only	Both	Neither
Myogenic tumors					
Rhabdomyosarcoma (RMS)					
Malignant Triton's tumor	31	0	0	31	0
Pulmonary blastoma	2	0	0	2	0
	1	0	0	1	0
Total	34	0	0	34	0
RMS simulators					
Ewing's sarcoma					
Lymphoma	4	0	0	0	4
Medulloblastoma	11	0	0	0	11
Neuroblastoma	5	0	0	0	5
Plasmacytoma	6	0	0	0	6
Rhabdoid tumor	1	0	0	0	1
Small cell carcinoma	1	0	0	0	1
	6	0	0	0	6
Total	34	0	0	0	34

studies demonstrated that optimal staining for desmin required proteolytic predigestion[32] (50 mg protease [Sigma Chemical Company, St. Louis, MO] and 200 mg $CaCl_2$ in 200 mL TRIS buffer, pH 7.2, for 30 minutes at 37 °C). In tissue from 25 cases (5 Zenker-fixed and 20 formalin-fixed) simultaneous staining for MSA, with and without protease digestion, showed that this digestion procedure did not improve MSA staining, regardless of the type of fixatives. In the case of anti-MSA, 50 mmol/L edetate (EDTA) was added to the primary antibody to eliminate nonspecific background staining.[45]

For each case, a set of consecutive sections consisting of those stained with hematoxylin and eosin, antidesmin, and anti-MSA was reviewed by two independent observers

TABLE 6. LEIOMYOSARCOMA AND SIMULATORS

	Number of Cases	Desmin Only	Actin Only	Both	Neither
Leiomyosarcoma (LMS)	22	3	4	7	8
LMS simulators					
Malignant fibrous histiocytoma	14	0	3	1	10
Neurofibrosarcoma	10	0	1	1	8
Glioblastoma multiform	8	0	0	3	5
Malignant mesothelioma	7	1	1	0	5
Liposarcoma	6	0	0	0	6
Osteosarcoma	6	0	0	0	6
Chondrosarcoma	3	0	0	0	3
Synovial sarcoma	3	0	0	0	3
Kaposi's sarcoma	2	0	0	0	2
Atypical fibroxanthoma	2	0	0	0	2
Angiosarcoma, breast	1	0	0	0	1
Dermatofibrosarcoma	1	0	0	0	1
Fibrosarcoma	1	0	0	0	1
Spindle cell carcinoma	1	0	0	0	1
Subtotal	65	1	5	5	54

TABLE 7. EPITHELIAL TUMORS

	Number of Cases	Positive Cases	
		Desmin	Actin
Carcinoma	34	0	0
Carcinoid tumor	2	0	0
Benign epithelial tumor	6	0	0
Total	42	0	0

for architectural and cytologic features of staining, the percentage of stained cells, and the similarity or difference of staining for these two antigens. Any discrepancy was settled by simultaneous examination.

RESULTS

Effects of Tissue Fixation

The type of fixative did not influence the staining for desmin significantly. Zenker's fixative decreased the intensity of staining and the number of cells stained for MSA. The adverse effect of Zenker's fixative on MSA staining was observed in normal and neoplastic tissues. In fact, as shown in Table 8, MSA staining was stronger than that of desmin in most RMS cases fixed in formalin, whereas the reverse was true for RMSs fixed in Zenker's solution. Decalcification lasting as long as 18 hours appeared to have no effect on the staining of normal duodenal smooth muscle cells by either antibody.

Normal Tissues

All samples from normal muscle (31 skeletal, 7 cardiac, and 42 nonvascular smooth muscle samples) were stained positively by both antibodies in virtually every cell (Table 1). Desmin was demonstrated focally in 100 of 196 samples of vascular smooth muscle, whereas MSA appeared diffusely in every sample. MSA also was expressed by pericytes (286 samples) but not by endothelial cells. Rare isolated spindle cells were stained by either antibody in several locations, including capsules of solid parenchymal organs such as the liver, lymph nodes, kidney, pancreas, and adrenal gland and lamina propria of the gastrointestinal tract.

Seven samples of decidual cells and two of nine samples of chorionic villous stromal cells focally exhibited MSA but not desmin (Figs. 1*A* and *B*). However, none of the 154 samples of nonmuscular tissues was reactive with either antibody (Table 2).

Myoepithelial Cells and Myofibroblasts

Myoepithelial cells in eccrine sweat glands (14 samples), salivary glands (6 samples), mammary glands (4 samples),

and bronchial glands (1 sample) consistently expressed MSA but not desmin (Table 3). However, none of three benign and one malignant mixed tumors of salivary glands exhibited MSA or desmin.

Muscle-specific actin was demonstrated in 46 of 62 reactive myofibroblast-containing lesions (3 of 3 atheromatous plaques, 39 of 49 tumor-associated desmoplastic lesions, 3 of 5 granulation tissues, 1 of 5 keloids). MSA also was present in 8 of 14 myofibroblast-containing neoplastic tissues (6 of 6 fibromatoses, 1 of 7 benign fibrous histiocytomas, 1 of 1 mammary myofibroblastoma). MSA-positive cells sometimes were abundant; thus, tumor-associated desmoplasia featuring a large number of MSA-positive cells might resemble a myogenic neoplasm superficially (Fig. 2*A*). Desmin was found in many fewer cases, and in fewer cells per case, than was MSA (Fig. 2*B*). In individual cells, staining for desmin was focal, whereas that for MSA was diffuse.

Benign Tumors

Of six leiomyomas, four (three uterine and one gastric) exhibited both MSA and desmin in approximately the same number of tumor cells; one gastric leiomyoma expressed only MSA and another gastric leiomyoma expressed only desmin (Table 4). In the tumor cells, the staining for MSA was diffuse, whereas desmin staining was globular. Web-like cytoplasmic staining was found with both antibodies in all four cardiac rhabdomyomas (Fig. 3). All 14 benign spindle cell tumors (4 meningiomas, 4 neurofibromas, 3 schwannomas, 2 hemangiomas, 1 cardiac myxoma); 6 benign epithelial tumors (*e.g.*, fibroadenomas); and 2 carcinoid tumors did not react with either antibody (Table 7).

TABLE 8. COMPARATIVE STAINING OF MYOGENIC TUMORS: THE INFLUENCE OF FIXATIVES

	Number of Cases	Staining Comparison		
		D > A	D = A	D < A
Formalin-fixed				
Rhabdomyosarcoma	13	1	6	6
Triton's tumor	2	1	0	1
Pulmonary blastoma	1	0	1	0
Rhabdomyoma	2	0	2	0
Leiomyosarcoma	22*	3	6	5
Leiomyoma	6	1	4	1
Zenker's-fixed				
Rhabdomyosarcoma	18	12	4	2
Rhabdomyoma	2	2	0	0

D = number of tumor cells positive for desmin; A = number of tumor cells positive for muscle-specific actin.

* Eight cases stained by neither desmin or actin.

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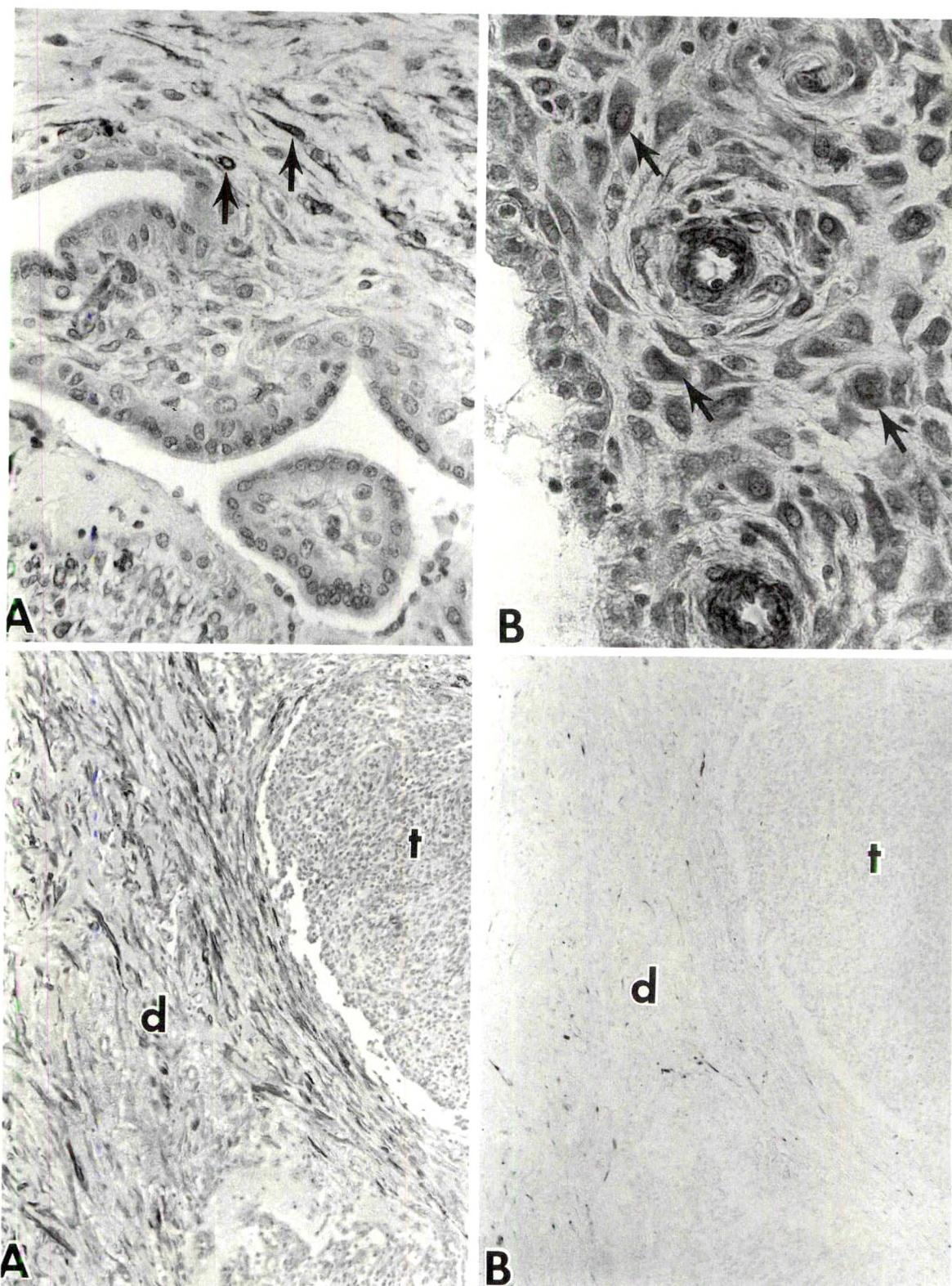
FIG. 1 (*upper*). *A.* Some stromal cells of chorionic villi (*arrows*) are stained positively for muscle-specific actin. ABC technique (×1,200). *B.* Muscle-specific actin is localized in the cytoplasm of some decidual cells (*arrows*). Note positive staining in the wall of decidual arterioles. An endometrial gland (*lower left*) is not stained. ABC technique (×1,200).

FIG. 2 (*lower*). *A.* Desmoplasia (*d*) associated with a sarcoma (*t*). Several myofibroblasts in the desmoplastic area are stained positively for muscle-specific actin. ABC technique (×1,200). *B.* A much smaller number of myofibroblasts in the same area is stained positively for desmin. ABC technique (×1,200).
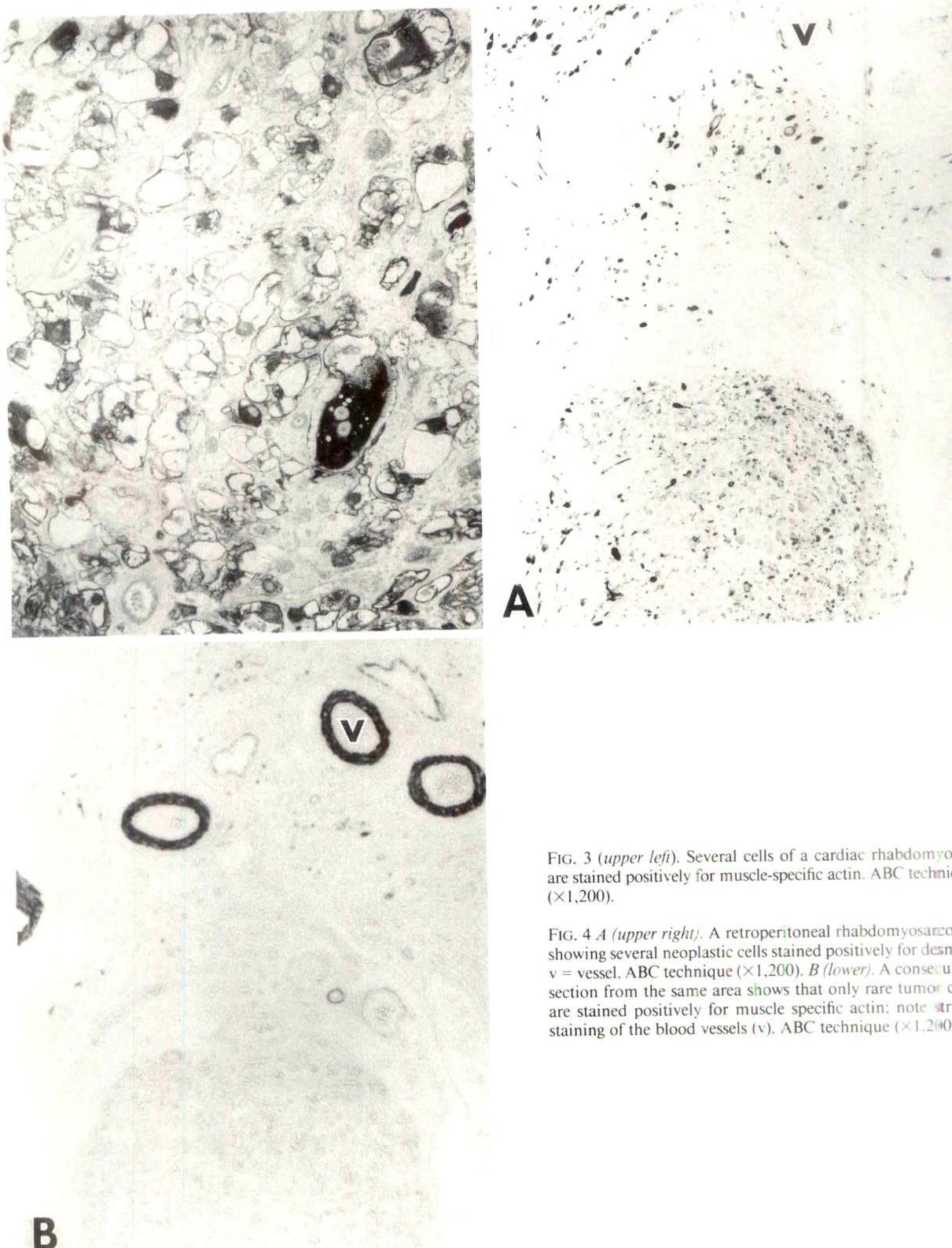
FIG. 3 (*upper left*). Several cells of a cardiac rhabdomyoma are stained positively for muscle-specific actin. ABC technique (×1,200).

FIG. 4 *A (upper right)*. A retroperitoneal rhabdomyosarcoma showing several neoplastic cells stained positively for desmin, v = vessel. ABC technique (×1,200). *B (lower)*. A consecutive section from the same area shows that only rare tumor cells are stained positively for muscle specific actin; note strong staining of the blood vessels (v). ABC technique (×1,200).

Malignant Tumors

All 31 RMSs expressed desmin and MSA regardless of location and histologic subtype (22 embryonal, 8 alveolar, and 1 pleomorphic) (Table 5). However, staining patterns were different for desmin and MSA. More than 60% of tumor cells stained for MSA and desmin in 12 and 19 cases, respectively. Rare tumor cells (<5%) stained for MSA and desmin in six and seven cases, respectively. Although the overall incidence of positive staining for desmin and MSA was roughly similar, case-by-case comparison of consecutive slides revealed a significant difference. Thus, there were 13 cases in which staining for desmin was remarkably more pronounced than that of MSA (Figs. 4A and B), whereas in 8 other cases, the reverse was true (Figs. 5A and B). Although the difference in desmin and MSA staining was explained, in part, by the intrinsically different expression of these antigens by tumor cells, the type of fixative was another important cause. Thus, tumors fixed in Zenker's solution frequently expressed less staining for MSA than for desmin (Table 8).

As seen in Table 6, among 22 LMSs (14 gastrointestinal tract, 7 soft tissue, and 1 uterine LMS), 7 expressed both markers, 3 demonstrated desmin only (Figs. 6A and B), and 4 expressed MSA only (Figs. 7A and B). All three LMSs that stained only for desmin were from the soft tissues (retroperitoneum, chest wall, and lower extremity, respectively). The four LMSs that expressed only MSA were from the soft tissues (three cases) and stomach (one case). All LMSs in which neither marker was demonstrated came from the gastrointestinal tract (three gastric, three small intestinal, and two colorectal tumors). In general, when both markers were demonstrated in the same tumors, the number of positive cells and the staining patterns were comparable.

All 34 malignant tumors containing predominantly small round undifferentiated cells (Table 5) were not reactive with either antibody. Among 65 malignant spindle cell tumors, focal MSA-immunoreactive cells were observed in 4 of 14 malignant fibrous histiocytomas (Fig. 8), 2 of 10 neurofibrosarcomas, 1 of 7 malignant mesotheliomas, and 3 of 8 cases of glioblastoma multiforme. Within the same group, one malignant fibrous histiocytoma, one neurofibrosarcoma, one malignant mesothelioma, and all three glioblastomas also exhibited desmin in corresponding areas of the sections (Figs. 9A and B). Neither antibody labeled neoplastic cells in 34 carcinomas from various sites.

DISCUSSION

In 1978 Vandekerckhove and associates[58] proposed that at least six different actin isotypes were expressed in higher mammalian tissues; four of them were found exclusively in muscular tissues, including alpha-skeletal, alpha-car-

diac, and alpha and gamma smooth muscle actins. The beta and gamma nonmuscular actins were the major cytoplasmic actins in cells other than those of muscle. Early antiactin antibodies were polyclonal and did not discriminate among various actin isotypes. Several studies have reported that most of these reagents possess low specificity and sensitivity for muscle differentiation,[8,12,13] but, in a study by Hashimoto and colleagues,[18] a "private" polyclonal antiserum against actin was believed to be diagnostically superior to other pertinent antibodies, including antidesmin.

Recently, many monoclonal antibodies have been described that recognize certain actin isotypes.[17,27,44,45,59-61] In 1985, Gown and co-workers[17] described a monoclonal antibody that recognized a common epitope of alpha and gamma smooth muscle actin isotypes. A single clinical study using this antibody showed that it was a good marker for myoepithelial cells and smooth muscle cells and showed positive staining in leiomyomas and LMSs but negative staining in carcinomas, melanomas, lymphomas, and sarcomas. In 1986, Skalli and associates[59] described an alpha-smooth muscle actin antibody that was reactive with normal smooth muscle cells, pericytes,[61] myoepithelial cells,[60] and reactive myofibroblasts[59] but did not label normal skeletal muscle and tumors thereof. Schurch and associates,[27] using the same antibody, observed a positive reaction in two of two leiomyomas, one of one vascular leiomyoma, and two of ten LMSs, whereas desmin was detected in five LMSs in the same group. These two studies suggested that the antibody in question is an excellent marker for myofibroblasts, myoepithelial cells, and pericytes but not LMS or normal and neoplastic skeletal muscles.

In 1988, Skalli and associates[60] introduced the monoclonal antibody alpha-SR-1, which is directed against alpha-skeletal and alpha-cardiac actin isotypes, and showed positive staining by this antibody in all 15 RMSs, with only 12 expressing desmin. It was also observed that selected antiactin antibodies also stained some cells other than myocytes. These cells have been reported in the deep ovarian cortical stroma and theca externa of secondary ovarian follicles,[62] alveolar soft part sarcoma,[63,64] infantile digital fibromatosis,[42,43,65] epithelioid sarcoma,[40] ovarian sclerosing stromal tumors,[41] and Kaposi's sarcoma.[37,38] It has been postulated that they represented either myofibroblasts or pericytes in these conditions. However, actin staining of unequivocal tumor cells has been reported in some cases of metastatic endometrial stromal sarcoma[66] and malignant peripheral nerve sheath tumor,[67] and in glomeruloid cells in a case of Wilms' tumor.[39] It has not been determined whether these findings indicate aberrant actin expression of the tumor cells or cross-reactivity of the antiactin antibodies.
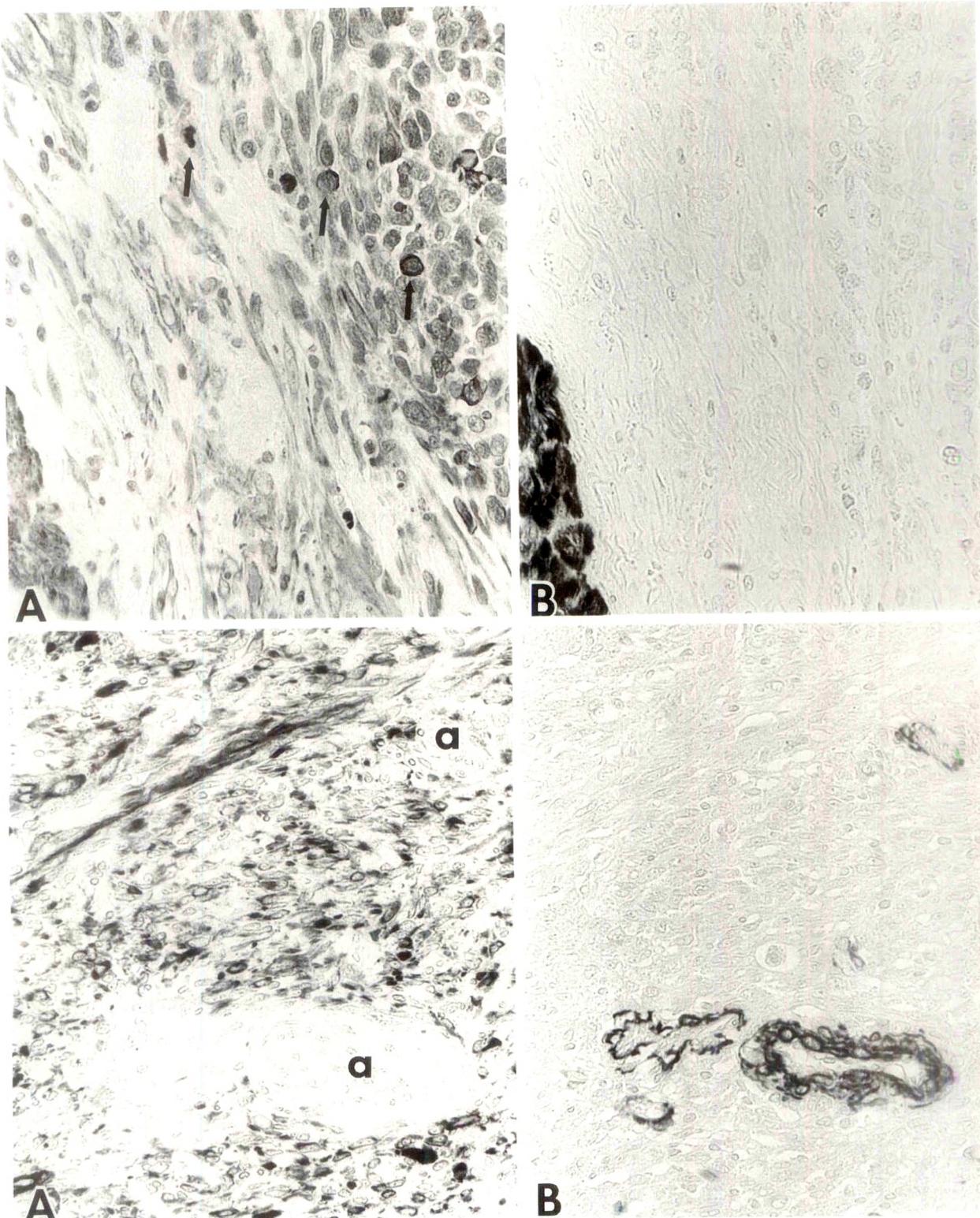
FIG. 5 (*upper*). *A.* Scrotal rhabdomyosarcoma showing some tumor cells that stained positively for muscle-specific actin (*arrows*). Note positive staining of the dartos muscle (*lower left*). ABC technique (×1,200). *B.* Consecutive section from the same area shows that none of the tumor cells is stained positively for desmin; however, the dartos muscle fibers (*lower left*) are strongly stained. ABC technique (×1,200).

FIG. 6 (*lower*). *A.* A soft tissue leiomyosarcoma displaying several tumor cells that stain for desmin; note negative staining of the blood vessels (*a*). ABC technique (×1,200). *B.* Consecutive section from the same area shows virtually no staining of tumor cells for muscle-specific actin. However, the blood vessel walls are strongly positive. ABC technique (×1,200).

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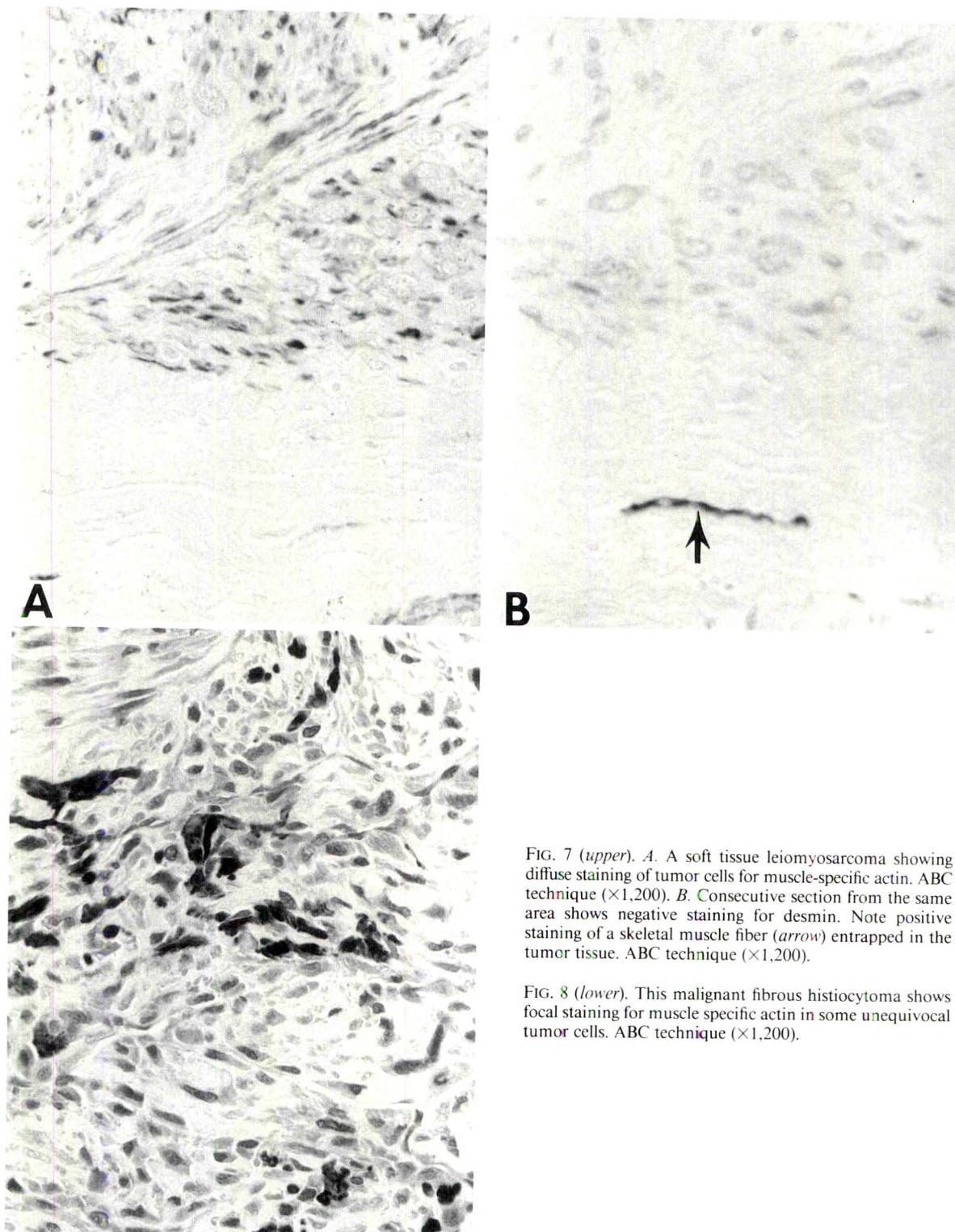
FIG. 7 (*upper*). *A*. A soft tissue leiomyosarcoma showing diffuse staining of tumor cells for muscle-specific actin. ABC technique (×1,200). *B*. Consecutive section from the same area shows negative staining for desmin. Note positive staining of a skeletal muscle fiber (*arrow*) entrapped in the tumor tissue. ABC technique (×1,200).

FIG. 8 (*lower*). This malignant fibrous histiocytoma shows focal staining for muscle specific actin in some unequivocal tumor cells. ABC technique (×1,200).

Almost all monoclonal antibodies against actin used in previous studies are not available commercially; one exception is that designated as HHF35, developed by Tsukada and colleagues.[45] This antibody recognizes a common epitope of alpha-skeletal, alpha-cardiac, and alpha and gamma smooth muscle actin isotypes, even in fixed, paraffin-embedded tissues.[51] Thus, it has practical application in diagnostic pathology.[46–48,50] Although the
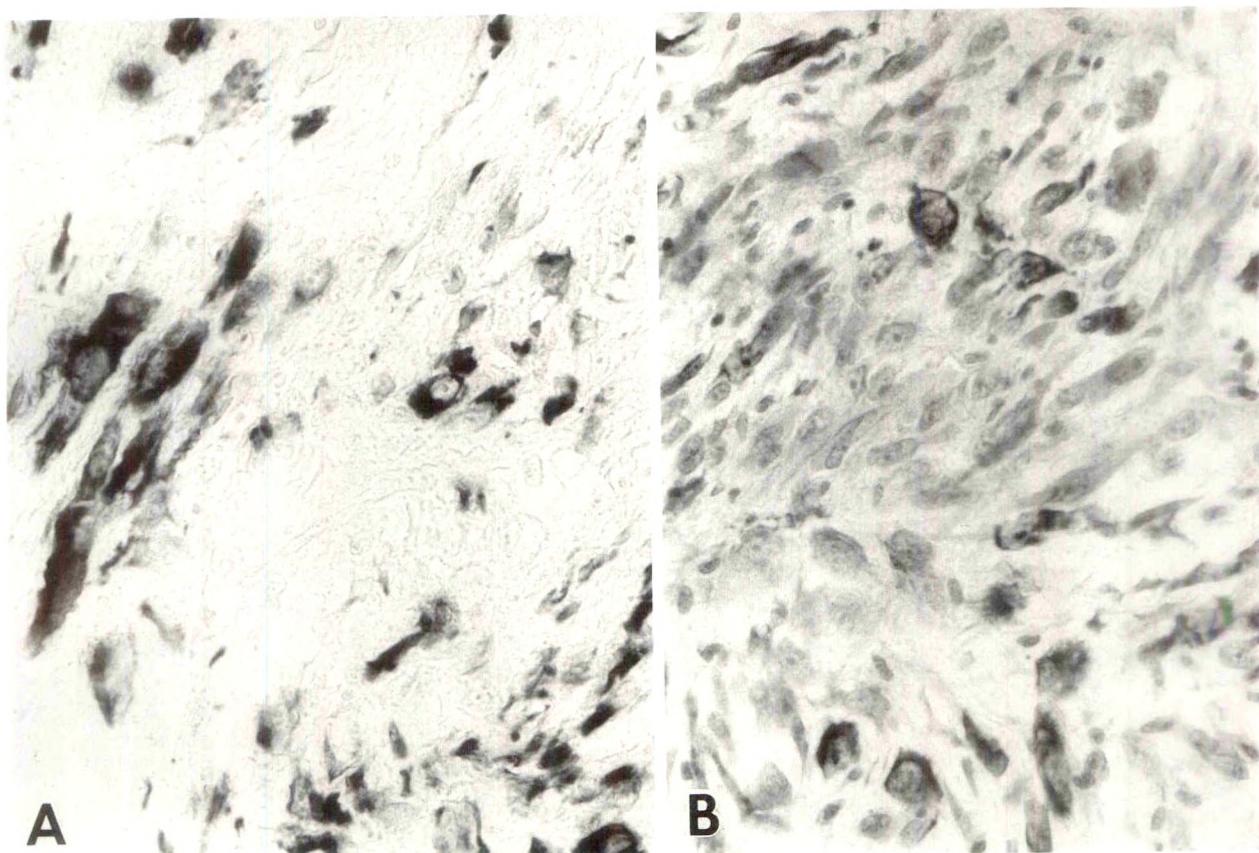
FIG. 9. This glioblastoma multiform shows focal staining of the tumor cells for both muscle-specific actin (*A*) and glial fibrillary acidic protein (*B*). ABC technique (×1,200).

HHF35 antibody has been used in several studies[45-51] to identify muscle differentiation in a variety of tissues, only one analysis has been devoted to a systematic evaluation of this antibody.[47] However, in this study, the effect of fixatives other than formalin was not evaluated; moreover, only tumor tissues were included. Our study has shown that, although satisfactory MSA staining was observed with formalin, B5, Bouin's, and ethanol fixation, Zenker's fixative drastically decreased the staining intensity. It is clear from this evaluation that a similarly deleterious effect of Zenker's fixative is not observed in desmin stains.

In addition to normal muscular tissues, MSA was present in myoepithelial cells, pericytes,[45,47] and cells in the capsules of several solid organs, including the liver, kidney, and spleen.[51] MSA was demonstrated in virtually every vascular smooth muscle cell, whereas desmin was expressed in only 53% of cases. Even in these positive cases the staining was focal. It is interesting that, in the same section of myometrium, antidesmin highlighted myometrial smooth muscle cells, but not those of arterial walls. These observations suggest that vascular and nonvascular smooth muscle cells may contain different cytoskeletal constituents. In fact, smooth muscle from different sources has been shown to exhibit major differences in actin isotypes.[68]

In our study, MSA was seen in decidual cells and some stromal cells in chorionic villi. To the best of our knowledge, this has not been observed previously.[45,47,51] MSA expression may represent the functional activity of these cells at certain stages of their development. Alternatively, such cells may be myofibroblasts, or they may be related to immature vascular smooth muscle cells or pericytes, intermingling with true stromal cells. Additional biochemical analysis of actin isotypes in this cell type may shed some light on this phenomenon.

Despite excellent immunoreactivity of the HHF35 antibody with myoepithelial cells in normal tissue, none of the five pleomorphic adenomas in our study expressed MSA. Nevertheless, Tsukada and associates[51] reported focal MSA expression in three of nine pleomorphic adenomas. Larger numbers of tumors with features of myoepithelial differentiation must be stained to explain this discrepancy. A similar situation exists for pericytes. Although the HHF35 antibody strongly stains pericytes, it

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has not been reported to label hemangiopericytomas.[47] These observations emphasize that neoplastic transformation may be associated with profound alteration in actin isotypes.

Although the histogenesis of myofibroblasts is still controversial, the presence of actin in these cells has been demonstrated biochemically and immunocytochemically.[69-73] Our study has shown that desmin and MSA are expressed by myofibroblasts, but MSA-positive cells of this type are much more numerous than desmin-positive myofibroblasts in reactive and neoplastic conditions. It was also mentioned that, despite adequate tissue preservation, some myofibroblasts did not stain for desmin or MSA. These observations may be explained by a recent finding by Skalli and associates[73] that cells with morphologic features of myofibroblasts were indeed heterogeneous and could be classified into four immunohistochemical phenotypes, according to their cytoskeletal compositions: cells were seen that expressed vimentin only; vimentin and alpha-smooth muscle actin; vimentin and desmin; and vimentin, alpha-smooth muscle actin, and desmin.

Our study supports previous reports stating that desmin and MSA are highly sensitive and specific markers for RMS. In contrast to other studies,[45-47,50,51] in which MSA uniformly was reported to be more sensitive than desmin, our study has shown that occasionally the reverse was true (Table 8); however, this observation could be ascribed to the deleterious effect of Zenker's fixation in most RMS cases included in this study.

In regard to LMS, our study has shown that, even when the tumor tissue was fixed in formalin, the number of MSA-positive cells could be equal to, smaller than, or larger than that of desmin-positive cells. Of more practical importance is the observation that the diagnostic sensitivity increased from 45% for desmin only, or from 50% for MSA only, to 64% if both markers were used. Using the same antibodies, Azumi and associates[46] and Miettinen[47] observed positive staining of 19 of 19 and 29 of 32 LMSs, respectively. The better results in these studies may be related in part to the observation that, in Azumi's study, only 2 cases of LMS from the gastrointestinal tract were included and, in Miettinen's study,[47] only LMSs from tissues other than the gastrointestinal tract were studied.

A problem encountered in our analysis as well as previous studies is the observation that a significant number of sarcomas with features of LMS did not express either desmin or MSA.[48] Most of these are from the gastrointestinal tract, as seen in 8 of 22 cases in our study and 5 of 11 cases in another series.[48] A possible explanation for this aberrant staining pattern may be related to the recent observation by Schurch and colleagues[27] that LMSs from different sites expressed different predominant actin iso-

types. In their study, most soft tissue and uterine LMSs contained predominantly alpha smooth muscle actin, but those from the gastrointestinal tract showed only beta and gamma nonmuscle actins and were devoid of alpha and gamma smooth muscle actins. The latter group of tumors is expected to show negative staining with the antibody HHF35, which is specific only for muscle actins. Alternatively, many gastrointestinal stromal cell tumors diagnosed as LMS may not be myogenic neoplasms; in fact, they may display neural differentiation.[48,74-76]

Tumors potentially confused with RMS, as shown in this series and previous studies, almost never showed positive staining for MSA or desmin. In contrast, rare cases of several types of spindle cell sarcoma, which may simulate LMS histologically, have been reported to express desmin, MSA, or both. Documented examples of such tumors include alveolar soft part sarcoma,[77] malignant fibrous histiocytoma,[47] clear cell sarcoma,[78] glomus tumor,[47,79] cutaneous pleomorphic fibroma,[80] capillary hemangioblastoma,[81] and mediastinal solitary fibrous tumor.[82]

In our study, a small number of sarcomas displayed rare, scattered, benign-appearing cells that were MSA positive and, less frequently, also desmin positive. These are regarded as reactive myofibroblasts, vascular smooth muscle cells, or pericytes entrapped in the tumor tissues. Even when these cases were eliminated, a few nonmyogenic spindle cell sarcomas (three malignant fibrous histiocytomas, one malignant schwannoma, three cases of glioblastoma multiforme, and one malignant mesothelioma) remained that clearly expressed MSA, desmin, or both, in clusters of unequivocal tumor cells. In all three cases of glioblastoma multiforme, the possibility of a sarcoma metastatic to the brain was excluded by the histologic features of the tumor, the absence of extracranial lesions on clinical examination, and positive staining for glial fibrillary acidic protein. Because double staining of single sections of the tumors was not done, we do not know whether glial fibrillary acidic protein and MSA were in the same or different tumor cells. In three cases of malignant fibrous histiocytoma with focal MSA staining, the possibility of a pleomorphic leiomyosarcoma was excluded by positive staining of several tumor cells for alpha-1-antitrypsin and the absence of myogenic differentiation by ultrastructural examination. These unusual staining reactions may, in fact, represent focal myogenic differentiation that does not reach a stage that is mature enough or extensive enough to be recognized by conventional light microscopic examination. Regardless of its histogenesis, this aberrant staining reaction indicates that caution must be used in the diagnostic interpretation of MSA and desmin stains.

Staining for Desmin and Muscle-Specific Actin

From this study, we conclude that MSA is expressed constantly in muscle cells, pericytes, decidual cells, and myoepithelial cells and frequently in myofibroblasts. We also determined that MSA and desmin are sensitive and specific markers for RMS. Although MSA is significantly more sensitive than desmin in formalin-fixed tissues, desmin is much more sensitive than MSA in Zenker-fixed tissues. For the diagnosis of LMS fixed in formalin, the sensitivities of MSA and desmin are roughly equal but rather low. Simultaneous staining for both antigens significantly increases the diagnostic yield but will not detect every case of LMS, especially those from the gastrointestinal tract. Finally, rare cases of nonmyogenic sarcoma may display unequivocal staining for MSA, desmin, or both.

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Original Article

Expression of a Polymorphic Epithelial Mucin Antigen Defined by the Monoclonal Antibody BC2 in Ovarian Carcinoma

Use of the BC2 Antibody for the Detection of Micrometastases

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The BC2 monoclonal antibody, which binds to an epitope on the peptide backbone of polymorphic epithelial mucin, was tested immunohistochemically for reactivity with epithelial ovarian carcinoma. This epitope was expressed in 90 of 91 malignant ovarian tumors; in 88% of these, more than 50% of the tumor cells expressed the epitope. In 94% of the positive tumors, the epitope was expressed on the cell membrane; in 56%, cytoplasmic expression was evident; and in 39%, secreted extracellular antigen was detected. Differences were not clearly discernible between dissimilar histotypes with respect to the percentage of cells expressing antigen and antigen localization. Thirteen of 19 benign ovarian cystadenomas also expressed the epitope, but staining was weak and restricted to the luminal surface of the cell membrane. A blind retrospective immunohistochemical analysis of all second-look laparotomy biopsy specimens from 20 patients also was performed. All four patients in whom microscopic disease was detected by standard pathologic assessment had BC2-positive metastases. Of seven patients in whom recurrent disease subsequently developed despite negative pathologic findings, four had biopsy specimens containing BC2 antigen–positive adenocarcinoma-like cells. Of the nine patients with negative results on operation and no recurrence, one had biopsy specimens containing BC2 antigen–positive adenocarcinoma-like cells. Mesothelial cells, although typically negative, expressed the epitope in one biopsy specimen, necessitating caution in the interpretation of positive cells. The BC2 antibody is reactive with most epithelial ovarian carcinomas and appears to be a useful tool for the detection of micrometastases. (Key words: Monoclonal antibody BC2; Ovarian cancer; Second-look laparotomy; Polymorphic epithelial mucin) Am J Clin Pathol 1991;96:46–52

Polymorphic epithelial mucins (PEMs) are glycoproteins consisting of a core protein of repeating 20 amino acid subunits, with mainly O-linked carbohydrate side chains. These molecules are found on the membranes of normal epithelial cells but are expressed strongly in epithelial tumors.[1] Many monoclonal antibodies have been raised to PEM using a wide variety of immunogens, resulting in antibodies that are reactive with various determinants and

show varied reactivity with benign and malignant tissue (for review see Zotter and associates[2]). Included in this group are antibodies to epithelial membrane antigen (EMA), which often are used in the diagnosis of epithelial malignancies.[3]

Several of these antibodies bind to epitopes on the repeating peptide core of PEM, including HMFG1, HMFG2, and SM3[4]; DF3[5]; BC1, BC2, and BC3[6]; and OM1.[7] The expression in carcinomas of the epitope defined by each antibody is dependent not only on the amount of PEM produced, but also on the state of glycosylation of the molecule. The BC2 monoclonal antibody recognizes an epitope that is highly expressed in breast cancer[8] and found in the ascites[9] and serum[10] of patients with ovarian cancer.

This study examines the expression of the BC2 epitope in ovarian carcinoma using immunohistochemistry, and it determines whether immunohistologic techniques could

improve detection of micrometastases in biopsy specimens obtained from patients with ovarian cancer who have had second-look laparotomy.

MATERIALS AND METHODS

Specimens and Analysis

Ninety-one ovarian carcinomas and 19 benign ovarian tumor specimens were selected retrospectively from the files of the Pathology Department at the Royal Brisbane Hospital. All tissue was formalin fixed and embedded in paraffin. A single block, which was considered to be representative of the tumor, was selected; and 5-μm sections were mounted on glass slides for immunohistochemical analysis. The ovarian tumors consisted of 35 serous, 17 endometrioid, 9 mucinous, 9 poorly differentiated, 8 clear cell, 1 mixed, and 1 adenosquamous carcinoma; 1 adenocarcinoma of no other specification; 4 serous, 5 mucinous, and 1 mixed tumor of low malignant potential; and 10 benign serous and 9 benign mucinous cystadenomas. Stained sections were analyzed, and the antigen was recorded as either being absent or present in less than 10%, 10–50%, or more than 50% of tumor cells. The cellular localization of antigens was recorded as being membranous, cytoplasmic, or both (membrane staining was defined as being luminal alone or on the entire membrane). The intensity of staining was not scored.

In addition, all biopsy specimens were obtained from 20 patients with ovarian cancer who underwent second-look laparotomy (range, 3–23 blocks per patient). Blocks were obtained from the files of the Pathology Departments of the Royal Brisbane and Princess Alexandria Hospitals and from two private pathology laboratories. Nine of these patients had no tumor detected in biopsy specimens and have no evidence of disease 15–87 months after operation (eight originally had stage III and one had stage IV disease). Seven patients had negative pathologic findings at operation but subsequently showed recurrence of tumor (five had stage III, one stage II, and one stage I disease). Disease was detected microscopically in four patients at operation (one had stage IV, two stage III, and one stage I disease). Serial sections were coded with a random number, and the code was not broken until all sections were assessed immunohistologically. Hematoxylin and eosin–stained sections also were prepared for this group. The entire section was inspected carefully for the presence of BC2-positive cells; if any were detected, their position, the approximate number of cells, their morphologic appearances, and the localization of the antigen were recorded. Detection of tumor cells by BC2 immunohistochemistry then was compared with the original assessment for each block from the pathology report.

Immunohistochemistry

The entire immunohistochemical procedure was performed at room temperature. Paraffin was removed from sections in xylene, followed by hydration in a graded series of ethanol and water solutions. Endogenous peroxidase activity was removed by a 30-minute incubation in 0.6% (volume/volume [v/v]) H_2O_2 in 80% (v/v) methanol, and nonspecific protein binding was blocked by a 10-minute incubation in 10% (v/v) fetal calf serum (FCS) in Dulbecco's phosphate-buffered saline (PBS) without Mg^{2+} and Ca^{2+}, pH 7.2. Slides were then laid flat in a humidified box and covered with monoclonal antibody for 40 minutes. The BC2 monoclonal antibody (Medical Innovations Ltd., Labrador, Australia) was used as undiluted culture supernatant with an immunoglobulin concentration of approximately 20 mg/L. Slides were washed in PBS (2 minutes), 0.5% (v/v) Nonidet P-40® (Sigma Chemical Company, St. Louis, MO) in PBS (2 minutes), and then PBS alone (two changes of 2 minutes), before being covered with peroxidase-conjugated rabbit antimouse immunoglobulin (DAKOPATTS, Denmark) diluted 1/50 in 5% FCS (v/v) in PBS. Sections were then washed as described above and covered with 1 g/L diaminobenzidine tetrachloride (Sigma) in PBS containing 0.03% (v/v) H_2O_2 for 6 minutes, washed in tap water, counterstained for 5 minutes with Mayer's hematoxylin, dehydrated with ethanol, cleared with xylene, and mounted in DPX® (BDH Chemicals, Kilsyth, Australia).

RESULTS

Expression of the BC2 Epitope in Ovarian Tumors

The PEM epitope recognized by the BC2 antibody was expressed by all ovarian carcinomas examined, except one mucinous tumor of low malignant potential (see Table 1). In 88% of malignant tumors, more than 50% of the cells expressed the epitope; in most of these, virtually all the tumor cells were stained. In 94% of the positive tumors, the epitope was expressed on the cell membrane; in 56%, cytoplasmic expression was evident; and, in 39%, secreted extracellular antigen was detected (see Table 2 and Fig. 1). Membrane staining usually was concentrated at the luminal surface of tumor cells, if lumina were present. Cytoplasmic antigen, where present, usually appeared diffusely throughout the cytoplasm, except in some well-differentiated tumors in which the antigen was localized, usually in the apical region of the cell. Extracellular antigen was found with similar frequency in tumors expressing cytoplasmic antigen and in neoplasms with only membrane staining.

Dissimilarities were not clearly discernible between different histotypes with respect to the percentage of cells expressing antigen and the antigen localization. For ex-

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TABLE 1. THE PROPORTION OF TUMOR CELLS EXPRESSING THE POLYMORPHIC EPITHELIAL MUCIN ANTIGEN DETECTED BY MONOCLONAL ANTIBODY BC2 IN OVARIAN EPITHELIAL TUMORS

Pathology	Cells Stained			
	0%	*<10%*	*10–50%*	*>50%*
Serous	0/35 (0)	1/35 (3)	2/35 (6)	32/35 (91)
Endometrioid	0/17 (0)	0/17 (0)	3/17 (18)	14/17 (82)
Mucinous	0/9 (0)	0/9 (0)	2/9 (22)	7/9 (78)
Poorly differentiated	0/9 (0)	0/9 (0)	1/9 (11)	8/9 (89)
Clear cell	0/8 (0)	0/8 (0)	0/8 (0)	8/8 (100)
Adenocarcinoma NOS	0/1 (0)	0/1 (0)	0/1 (0)	1/1 (100)
Mixed	0/1 (0)	0/1 (0)	0/1 (0)	1/1 (100)
Adenosquamous	0/1 (0)	0/1 (0)	0/1 (0)	1/1 (100)
Serous LMP	0/4 (0)	0/4 (0)	0/4 (0)	4/4 (100)
Mucinous LMP	1/5 (20)	1/5 (20)	0/5 (0)	3/5 (60)
Mixed LMP	0/1 (0)	0/1 (0)	0/1 (0)	1/1 (100)
Benign serous	3/10 (30)	0/10 (0)	0/10 (0)	7/10 (70)
Benign mucinous	3/9 (33)	2/9 (22)	2/9 (22)	2/9 (22)

NOS = no other specification; LMP = low malignant potential; numbers in parentheses are percentages.

ample, although cytoplasmic expression was relatively common in clear cell carcinoma and relatively uncommon in mucinous cystadenocarcinoma, the numbers of each tumor type were insufficient to indicate statistical significance. Sixty-eight percent of ovarian cystadenomas also expressed the epitope, but in these tumors the staining was weak and typically restricted to the luminal surface of the cell membrane. In some cases, localized granular cytoplasmic staining was observed; no extracellular staining was seen in these tumors. The antigen appeared to be expressed by a higher proportion of serous than mucinous cystadenoma cells.

Second-Look Laparotomy and Biopsy

Results from the immunohistochemical analysis of the second-look biopsy specimens are presented in Table 3. Examples of metastases detected by immunohistochemical analysis are shown in Figure 2. Of the four patients with microscopic disease detected by standard pathologic assessment, two had a greater number of positive biopsy specimens found by immunohistochemical analysis, one had the same number, and one had less. Of the seven patients in whom recurrent tumor subsequently developed despite negative pathologic findings, four had biopsy specimens containing BC2 antigen–positive cells. These were restricted to micrometastases in one biopsy specimen from each patient. The tissues involved were a lymph node, an obturator lymph node, a paraaortic lymph node, and an ovarian pedicle. In three of these patients, the morphologic characteristics of the stained cells were consistent with those of ovarian carcinoma, but, in the other, the number of stained cells was small and their morphologic characteristics were indeterminate.

Of the nine patients with negative pathologic findings

and no recurrence, one had biopsy specimens containing BC2 antigen–positive adenocarcinoma-like cells. This patient had three positive biopsy specimens (paracolic gutter, diaphragm, and omentum). One patient had a group of cells—probably identified correctly as mesothelial in the original pathology report—that showed cytoplasmic staining; this constituted a false-positive result. Nonmalignant epithelial tissue encountered in these biopsy specimens included a mucinous cystadenoma of the appendix that showed weak luminal membrane staining in less than 50% of the cells; mucosa of the small bowel that showed weak luminal membrane staining in less than 1% of cells; dysplastic cervical epithelium, which was

TABLE 2. THE LOCALIZATION OF THE POLYMORPHIC EPITHELIAL MUCIN ANTIGEN DETECTED BY MONOCLONAL ANTIBODY BC2 IN OVARIAN EPITHELIAL TUMORS

Pathology	Number Detected		
	Membrane	*Cytoplasm*	*Extracellular*
Serous	33/35 (94)	18/35 (51)	9/35 (26)
Endometrioid	15/17 (88)	10/17 (59)	5/17 (29)
Mucinous	8/9 (89)	3/9 (33)	4/9 (44)
Poorly differentiated	9/9 (100)	6/9 (67)	4/9 (44)
Clear cell	8/8 (100)	7/8 (88)	4/8 (50)
Adenocarcinoma NOS	1/1 (100)	0/1 (0)	0/1 (0)
Mixed	1/1 (100)	1/1 (100)	0/1 (0)
Adenosquamous	1/1 (100)	1/1 (100)	1/1 (100)
Serous LMP	4/4 (100)	1/4 (25)	2/4 (50)
Mucinous LMP	4/4 (100)	2/4 (50)	0/4 (0)
Mixed LMP	1/1 (100)	1/1 (100)	1/1 (100)
Benign serous	7/7 (100)	2/7 (29)	0/7 (0)
Benign mucinous	5/9 (56)	2/9 (22)	0/9 (0)

NOS = no other specification; LMP = low malignant potential; numbers in parentheses are percentages.
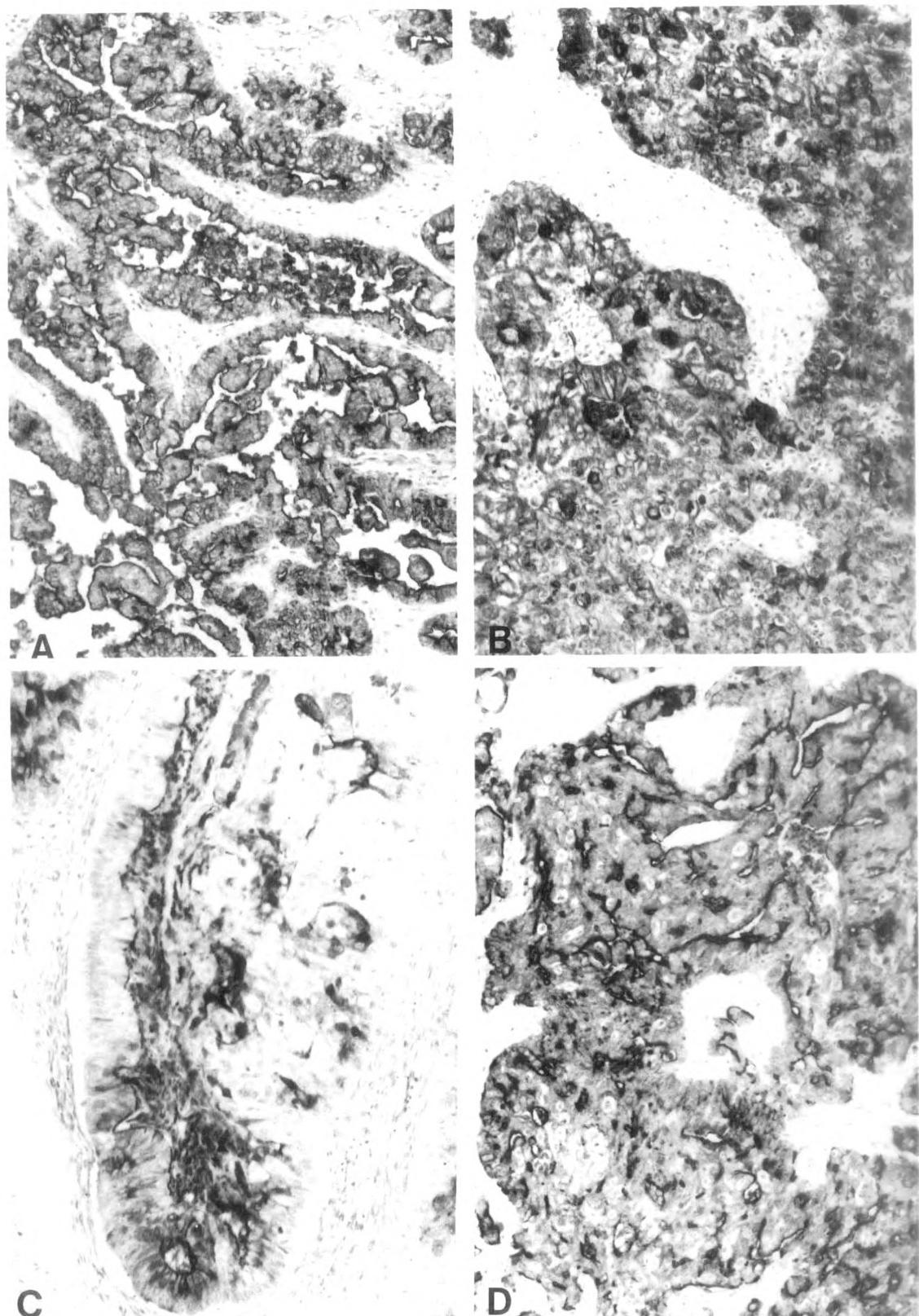
FIG. 1. Demonstration of the antigen recognized by the monoclonal antibody BC2 in different histotypes of routinely fixed, paraffin-embedded ovarian epithelial cancers. *A.* Luminal membrane staining in a moderately differentiated serous cystadenocarcinoma (×100). *B.* Cytoplasmic staining in a clear cell carcinoma (×100). *C.* Luminal membrane, cytoplasmic, and extracellular staining in a mucinous cystadenocarcinoma (×100). *D.* Luminal membrane and cytoplasmic staining in an endometrioid carcinoma (×100).

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TABLE 3. COMPARISON OF IMMUNOHISTOCHEMICAL (IHC) AND STANDARD PATHOLOGICAL DETECTION OF METASTASES IN BIOPSY SPECIMENS OBTAINED FROM PATIENTS UNDERGOING SECOND-LOOK LAPAROTOMY FOR EPITHELIAL OVARIAN CANCER

Patients	Pathology	IHC
Negative Operation/No Recurrence		
1	0/18	3/18
2	0/20	0/20
3	0/14	0/14
4	0/11	0/11
5	0/10	0/10
6	0/13	0/13
7	0/10	0/10
8	0/10	0/10
9	0/26	0/26

Patients	Pathology	IHC
Negative Operation/Recurrence		
10	0/23	1/23
11	0/12	0/12
12	0/13	0/13
13	0/15	1/15*
14	0/7	0/7
15	0/16	1/16
16	0/16	1/16
Positive operation		
17	3/3	3/3
18	1/22	3/22
19	13/17	10/17
20	7/22	3/22

The number of biopsies containing tumor/total number of biopsies is shown.

* Small number of cells whose morphology was not clear.

weakly positive; and endometrial epithelium, which was strongly positive. In addition, some bundles of smooth muscle and nervous tissue also showed weak diffuse cytoplasmic staining.

DISCUSSION

As expected, the BC2 epitope was expressed strongly in all malignant ovarian tumors in this series. In benign tumors, the epitope usually was expressed weakly only on the luminal membrane surface, whereas, in malignant cells, expression on the cell membrane was enhanced and expression in the cytoplasm and extracellular space was often evident. This difference in expression probably results from an increase in PEM production, a change in glycosylation, or a combination of both factors. Although this pattern of expression is not specific for ovarian malignancy, the characteristic staining patterns and the high frequency of BC2 expression suggest that this antibody would be useful for the detection of micrometastases. This epitope is expressed more strongly and universally in paraffin sections of ovarian carcinoma than epitopes recognized by other PEM peptide-backbone–binding monoclonal antibodies, such as BC3 and OM1, and the PEM carbohydrate binding antibody F36/22, which were tested previously in this laboratory.[11] Although the HMFG2 antibody has not been tested on the same sections, our previous findings suggest that the BC2 epitope is expressed more universally in ovarian carcinoma than that of HMFG2, particularly in poorly differentiated tumors.[12]

Although the use of second-look laparotomy in the treatment of ovarian cancer remains somewhat controversial, existing metastases must be detected if the procedure is to be helpful. Patients with microscopic disease are most likely to benefit from continued chemotherapy.[13] In a collation of published results,[13] it was calculated that recurrent disease subsequently developed in 28% of 265 patients who originally had stage III or IV disease and were found to be free of disease at second-look laparotomy. Incorrect classification of disease status at the time of second-look laparotomy results from the inability to detect metastases in biopsy specimens or, perhaps more likely, an inability to perform biopsies at all possible sites of metastatic disease.

The current study demonstrated that immunohistochemical studies with the BC2 monoclonal antibody could detect small groups of metastatic tumor cells that otherwise were not distinguished readily from surrounding benign tissue. Roughly half of the patients with negative second-look results in whom recurrent disease subsequently developed had immunohistochemically detected micrometastases. However, one of nine patients with negative second-look results also had micrometastases, despite a disease-free follow-up of 42 months and no prior postoperative chemotherapy.

Although many groups of mesothelial cells seen in biopsy specimens in this series were not stained by the antibody, the presence of positive mesothelium in at least one specimen necessitates caution in the interpretation of positive cells; therefore, morphologic assessment of all positive cells is essential for correct interpretation. The possibility of BC2-positive mesothelial cells probably also precludes the use of this antibody to identify tumor cells in peritoneal washings obtained from these patients, although this application was not tested in this retrospective study. However, this epitope appears to have a lower level of expression in benign epithelium and mesothelial cells than that of cytokeratins, which often are used as markers for adenocarcinoma cells.[14,15] A direct comparison of BC2 with HMFG2, and the E29 monoclonal antibody used to detect EMA, is required to determine differences in the expression of the epitopes recognized by these antibodies in benign and malignant epithelial tissues. Although the E29 epitope may be present in the same antigen as that recognized by HMFG2,[16] and therefore also BC2, this does not equate with identical immunohistochemical expression.[11]

In conclusion, the epitope defined by the BC2 antibody is expressed strongly in routine paraffin sections of ovarian carcinoma. Because of this strong expression and a lower antigen density in benign tissue, this antibody can be used to highlight micrometastases immunohistochemically. This may be most useful in lymph nodes, in which the
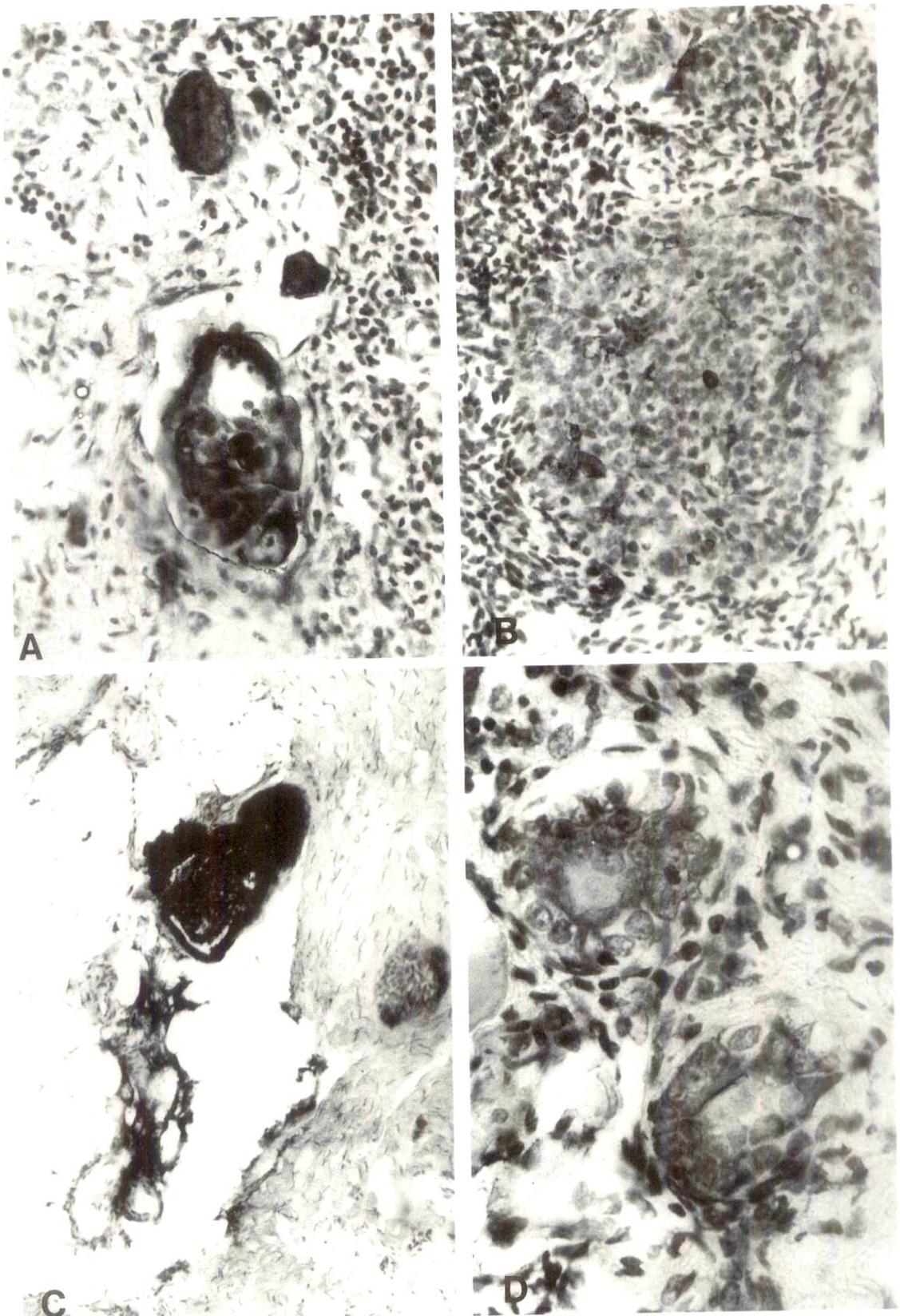
FIG. 2. Demonstration of the antigen recognized by the monoclonal antibody BC2 in biopsy specimens obtained from patients undergoing second-look laparotomy after treatment for ovarian epithelial cancer. *A.* Small clumps of antigen-positive cells in a para-aortic lymph node from a patient (BS) who suffered recurrent disease after negative second-look findings (×250). *B.* A clump of luminal membrane antigen-positive cells in a lymph node from a patient (JC) who suffered recurrent disease after negative second-look findings (×250). *C.* A clump of strongly antigen-positive cells in the ovarian pedicle from a patient (ST) who suffered recurrent disease after negative second-look findings (×100). *D.* Two small foci of luminal membrane antigen-positive cells in the omental tissue of a patient (NB) with negative second-look findings and no evidence of recurrent disease 42 months after surgery (×400).

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plethora of nuclear morphotypes presents a significant challenge to the pathologist searching for micrometastases. Detection of such lesions would allow for more appropriate selection of treatment, after second-look laparotomy. Immunohistochemical identification of micrometastases in lymph nodes also would ensure correct staging after lymphadenectomy, during primary surgery.

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Original Article

Comparison of Monoclonal Immunocytochemical and Immunoenzymatic Methods for Steroid Receptor Evaluation in Breast Cancer

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The production of monoclonal antibodies against estrogen receptor (ER) and progesterone receptor (PR) has permitted the development of the enzyme immunoassay (EIA) and immunocytochemical assay (ICA) for steroid receptor determination. The results obtained with these two techniques, using the same monoclonal antibodies, were compared in a large series of breast carcinomas (187 for ER and 100 for PR). The correlation between these methods was significant for ER (r_s = 0.54) and PR (r_s = 0.55) ($P < 0.001$) but was lost when the receptor concentrations determined by EIA were ≤ 15 and ≤ 30 fmol/mg protein for ER and PR, respectively. When these values are considered as cutoffs, the concordance between the two methods was 84.5% for ER and 73% for PR. An analysis of discordant results revealed that low epithelial cellularity generally was present in ICA-positive, EIA-negative specimens, whereas only focal positivity with ICA, or positivity of only normal peripheral mammary ducts and lobules, frequently was found in ICA-negative, EIA-positive tumors. In conclusion, there is good correlation between the results obtained by EIA and ICA methods for detection of ER and PR. The authors suggest that biochemical and histochemical methods for steroid receptors could be considered complementary and used together for the analysis of breast cancer. (Key words: Estrogen receptor; Progesterone receptor; Immunocytochemistry; Monoclonal antibodies; Breast cancer) Am J Clin Pathol 1991;96:53–58

The evaluation of estrogen (ER) and progesterone (PR) receptor content in breast cancer is an important factor in assessing the value of endocrine therapy.[1–3] ER and PR determination currently is accomplished with the dextran-coated charcoal (DCC) biochemical method.[4] This procedure is believed to be accurate, but it has various shortcomings, such as the use of radioactive materials, and it requires relatively large tissue samples and several days for the analysis to be completed.[5] These inconveniences may be overcome with immunoenzymatic procedures.

The production of monoclonal antibodies to ER by King and Greene,[6] and, more recently to PR, by Greene and Press,[7] has permitted the development of an enzyme immunoassay (EIA) that is based on the direct recognition of steroid receptor molecules. The ER/PR EIA is commercially available in kit form (ER-EIA and PR-EIA; Abbott Laboratories, North Chicago, IL). The immunoenzymatic method requires (as do all biochemical techniques) the homogenization of tissue samples, with loss of cell integrity, so that it is impossible to assess the degree of heterogeneity in receptor distribution within the tumor.

In contrast, the direct recognition of ER and PR in tissue sections of carcinomas is possible with the same monoclonal antibodies, using the immunocytochemical assay (ICA) (ER-ICA and PR-ICA; Abbott).[8–12] Several authors have shown a good correlation between the EIA[13–16] or ICA[8,9,17–19] and DCC for ER and PR evaluations in breast cancers. A relationship also has been found between the results obtained by EIA and ICA for ER

analysis.[20,21] Thus far, no data are available on the relationship between PR immunostaining and PR content by EIA.

In this study, we compared results of EIA and ICA in the evaluation of ER and PR in breast cancer, using the same monoclonal antibodies raised against ER and PR. The cutoff value of ER and PR positivity by EIA was determined, and the differences that emerged with the use of these two methods are discussed.

MATERIALS AND METHODS

Patients

We examined 187 patients, aged 27–88 (mean, 58.9 ± 13.1) years, who underwent biopsy or radical mastectomy for breast cancer.

Histology

Tumors were classified histologically according to the World Health Organization Classification[22] for mammary neoplasms.

Tissue Preparation

Breast cancer specimens were received from the operating room within 30 minutes of excision. The tumors were divided into three parts: the first was submitted for routine histopathologic examination; the second was frozen rapidly in liquid nitrogen and stored in OCT Tissue Tek® at −70 °C for ICA of ER and PR; and the last portion was stored in liquid nitrogen for EIA of ER and PR.

Data from ER analysis were available for all 187 tumors, whereas PRs were examined in 100 of them.

Immunocytochemical Assay

Estrogen receptor and PR immunostaining was performed according to the procedure of King and associates[8] and in accordance with the manufacturer's instructions, using anti-ER and -PR monoclonal antibodies that are commercially available in kit form (Abbott). Four frozen sections from the same specimen were fixed in 3.7% formaldehyde–phosphate-buffered saline (PBS) solution (pH 7.4), cold methanol, and acetone, and treated with normal goat serum to prevent nonspecific binding of subsequent reagents. Two samples were incubated with rat monoclonal antibody to human ER or PR, and the others were incubated with normal rat serum. The slides were incubated with a goat antirat antibody, and the rat peroxidase–antiperoxidase (PAP) complex then was added to each sample. A chromogen substrate solution containing hydrogen peroxide and diaminobenzidine was added to each sample. The reaction of peroxidase with hydrogen peroxide converted the diaminobenzidine to an insoluble brown product that permitted the visualization of the ER– or PR–monoclonal antibody complexes.

Results were evaluated as the percentage of positive cells. Values greater than 10% were considered positive for ER and PR[9,12] (Fig. 1).

Immunoenzymatic Assay

Estrogen receptor and PR were assayed immunoenzymatically according to the manufacturer's instructions (Abbott). Briefly, frozen neoplastic tissue was pulverized and homogenized in 10 volumes of buffer (TRIS buffer 10 nmol/L, EDTA 1.5 nmol/L, Na_2MoO_4 5 nmol/L, monothioglycerol 1 nmol/L, pH 7.4). The cytosol obtained by ultracentrifugation (one hour at 105.000 × g) was incubated with beads coated with an antireceptor monoclonal antibody. Unbound material present in the cytosol was removed by aspirating the fluid and washing the beads.

FIG. 1. Ductal infiltrating carcinomas with marked positivity for (*A*) ER-ICA and (*B*) PR-ICA (×250).

The presence of immune reactions in the standards, controls, and cytosol samples was detected by a second monoclonal antireceptor antibody conjugated with horseradish peroxidase. The chromogenic substrate was represented by ortophenylendiamine, developing a color that was analyzed by a spectrophotometer at 492 nm and allowed a measurement of bound receptor conjugate, expressed as femtomoles/milligram of protein. The sensitivities of ER-EIA and PR-EIA reported by the manufacturer were 1.0 and 1.5 fmol/mg protein, respectively.

Statistical Analysis

The correlation between the percentage of positive cells by ICA and the concentrations of the receptors by EIA

was analyzed by a nonparametric method (Spearman's correlation coefficient). Chi-square cross-correlation analysis was used to obtain the results in the tables.

RESULTS

The histotypes of the carcinomas were as follows: 161 (86%) ductal infiltrating, 16 (8%) lobular infiltrating, 3 (2%) medullary, 5 (3%) mucinous, 1 (0.5%) inflammatory, and 1 (0.5%) papillary.

Correlation Between ER-ICA and ER-EIA

One hundred twenty-nine (69%) of 187 tumors were ER-ICA positive (ER-ICA+). A good correlation (r_s
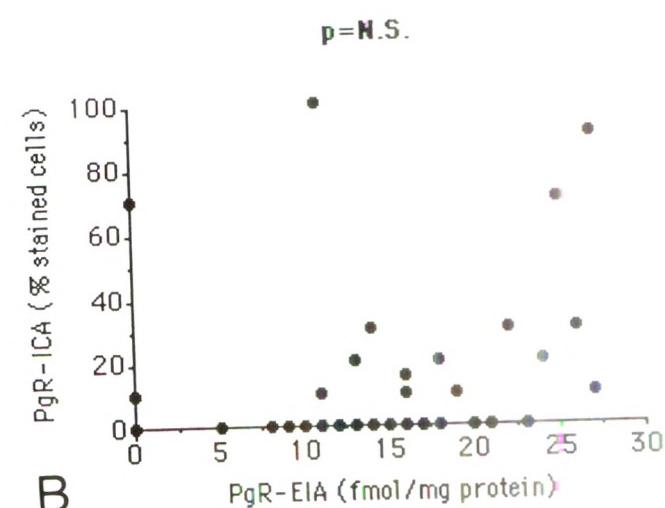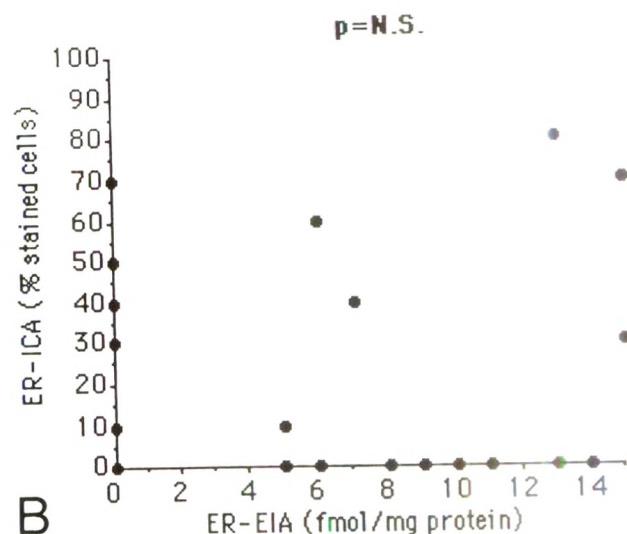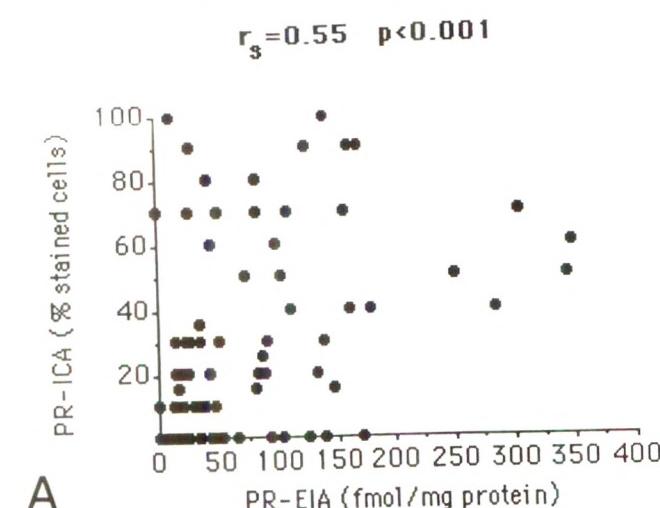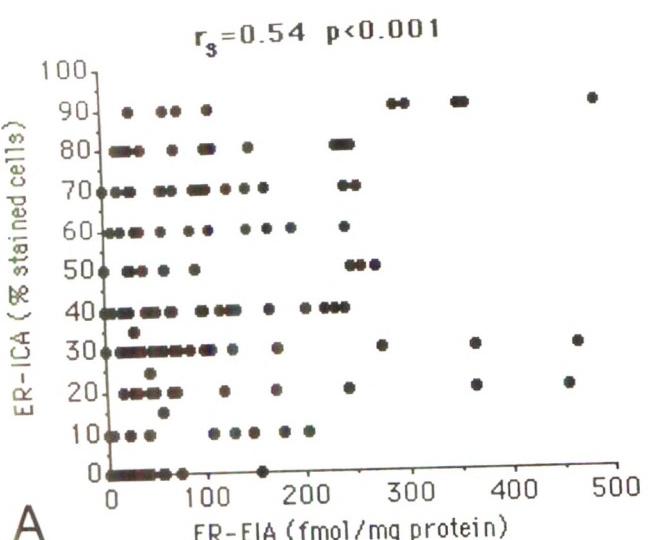
FIG. 2 (*upper*). *A.* Relationship between the percentage of positive cells by ER-ICA and the concentration of ER by EIA. *B.* The correlation was lost taking into account only the specimens with ER concentrations of ≤15 fmol/mg protein by EIA.

FIG. 3 (*lower*). *A.* Relationship between the percentage of positive cells by PR-ICA and the concentration of PR by EIA. *B.* The correlation was lost taking into account only the specimens with PR concentrations of ≤30 fmol/mg protein by EIA.

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= 0.54; *P* < 0.001) was demonstrated between the percentage of cells positive by ER-ICA and the concentration of ER by EIA (Fig. 2*A*). This correlation was lost when only those specimens with ER concentrations of ≤15 fmol/mg protein by EIA were considered (Fig. 2*B*). In this series, 136 tumors (72.7%) had ER values of >15 fmol/ mg protein, and 118 of the tumors (86.8%) had positive results by ICA. When these values are taken into account, the concordance between the results obtained with the two methods was 84.5% (*P* < 0.0001) (Table 1); discordant results were obtained in 29 specimens.

Correlation Between PR-ICA and PR-EIA

Forty-eight of 100 tumors were PR-ICA positive (PR-ICA+). The positive relationship between PR concentrations by EIA and the percentage of positive cells by ICA was high (r$_s$ = 0.55; *P* < 0.001) (Fig. 3*A*). The EIA and ICA results were found to be unrelated when only the specimens with PR concentrations of ≤30 fmol/mg protein by EIA were analyzed (Fig. 3*B*). Fifty-three tumors had PR-EIA concentrations of >30 fmol/mg protein, and 37 (69.8%) had positive results by ICA. When these values are considered, the concordance between the two methods was 73% (*P* < 0.0001); discordant results were obtained in 27 specimens (Table 2).

Analysis of Discrepancies Between ICA and EIA Results

Estrogen Receptor. Eleven tumors (5.9%) were ICA positive (ICA+), EIA negative (EIA−). Histologic examination of this group showed significant stromal proliferation associated with very low epithelial cell numbers in eight specimens (Table 3). Eighteen tumors (9.6%) were ICA negative (ICA−), EIA positive (EIA+). In this group, ten specimens showed focal and weak nuclear staining. Five tumors demonstrated immunostaining in normal epithelium adjacent to ICA− malignant cells, and nonspecific staining was found in one tumor (Table 3).

Progesterone Receptor. Eleven neoplasms were ICA+,

TABLE 1. DISTRIBUTION OF ER ACCORDING TO ICA AND EIA METHODS

	ICA		
	+	−	
EIA			
+	118	18	136
−	11	40	51
Totals	129	58	187

Concordance: 158/187 (84.5%). *P* < 0.0001.
ER-ICA: positive if >10% stained cells.
ER-EIA: positive if >15 fmol/mg protein.

TABLE 2. DISTRIBUTION OF PR ACCORDING TO ICA AND EIA METHODS

	ICA		
	+	−	
EIA			
+	37	16	53
−	11	36	47
Totals	48	52	100

Concordance: 73/100. *P* < 0.0001.
PR-ICA: positive if >10% stained cells.
PR-EIA: positive if >30 fmol/mg protein.

EIA−, of which 3 showed marked fibrosis with low epithelial cell content. In four specimens, the prevalence of ICA+ cells was low (15–30%), and, in one tumor, normal ducts and lobules that lacked immunostaining were scattered among the malignant tissue (Table 3). Sixteen specimens were ICA−, EIA+. Six tumors showed positive staining in the normal breast epithelium adjacent to neoplasms, five exhibited nonspecific staining, one showed focal staining, and another displayed a low percentage (10%) of positive cells, but these had intense nuclear staining (Table 3).

DISCUSSION

Good correlation has been demonstrated recently between immunoenzymatic and DCC assays for ER and PR.[13-16] It is interesting that the concordance of the results obtained from these methods is considered to be excellent for cytosol samples with steroid receptor values of >10 fmol/mg protein.[16,23] Nevertheless, the cutoff value for ER and PR positivity in the EIA procedure has not been determined, with suggestions ranging from 10 to 50 fmol/ mg protein.[13-16,20]

Several studies also have shown a correlation between immunocytochemical and biochemical DCC results, in the evaluation of steroid receptors in breast cancer.[8,9,17-19] Furthermore, various authors have demonstrated a relationship between the results obtained by EIA and ICA in ER evaluations.[20,21] Thus far, similar data are not available for PR.

In this study, we examined the association between EIA and ICA for ER and PR determinations, using the same monoclonal antibodies for both techniques. We must point out that the receptor site reacting with the monoclonal antibody is not necessarily the same as that binding the corresponding hormone.[24] This concept is important in the evaluation of breast tumors in premenopausal patients, whose receptors may be occupied by endogenous hormones.

TABLE 3. ANALYSIS OF DISCORDANT CASES

	n (ER)	n (PR)	Possible Cause of Discordance
ICA+, EIA−	8	3	Low epithelial cellularity
		4	Low positivity at ICA
		1	Tumor cells scattered among normal ICA-negative structures
	3	3	Unexplained
Totals	11	11	
ICA−, EIA+	10	1	Focal positivity at ICA
		1	Low (10%) positivity with marked staining intensity at ICA
	5	6	Positivity of normal ducts and lobules
	1	5	Aspecific (cytoplasmic) staining
	2	3	Unexplained
Totals	18	16	

In our series, the distribution of ER, by EIA or ICA methods, was similar to that reported by others. We also confirmed a good relationship between the results obtained by EIA and ICA in the evaluation of ER status. Furthermore, using various concentrations of ER (EIA), we have demonstrated that this correlation is lost when one only considers specimens with ER values of ≤ 15 fmol/mg protein.

The results reported for PR determinations by EIA and DCC are contradictory. Noguchi and associates[15] demonstrated an excellent correlation between these techniques (concordance, 91.4%). In contrast, Smyth and associates[25] and Raam and Vrabel[26] reported that they had obtained false-positive results with the EIA method, with a cutoff value of 10 fmol/mg protein for both DCC and EIA. Furthermore, Raam and Vrabel have reported that the quantity of PR sites in cytosol samples measured by DCC did not correlate well with PR values as measured by EIA.[26]

Using the same monoclonal antibody provided by Abbott Laboratories, Pertschuk and associates[12] documented a good correlation between the PR values as measured by ICA and DCC. Data are not available for PR on the relationship between EIA and ICA.

In our study, we observed a prevalence of PR-EIA+ and PR-ICA+ tumors that is similar to that reported by others, and a good correlation was found between these techniques. In analyses of various concentrations of PR in the EIA method, this correlation was lost when the only tumors considered had a PR content of ≤ 30 fmol/mg protein.

The possible causes of discordance between the results obtained by EIA and ICA were examined. A low epithelial

cellularity was generally present in ICA+, EIA− specimens. Focal positivity by ICA and the positivity of normal peripheral ducts or lobules were found frequently in ICA−, EIA+ specimens. In six EIA+ cases (five PR+ and one ER+), nonspecific cytoplasmic staining was identified by ICA. This finding may be explained by implicating the presence of unquenched endogenous peroxidase, causing nonspecific reactivity with the detection system in the two methods.

In this study, we demonstrated that ICA and EIA methods show good correlation in the evaluation of ER and PR status in breast cancers. Regression analysis indicated that this relationship was lost with ER and PR concentrations of ≤ 15 and ≤ 30 fmol/mg protein, respectively. Therefore, we suggest that these values be used as cutoffs for steroid receptor positivity in breast cancer when an EIA is used. The analysis of discordant cases indicates the importance of the evaluation of tumor cellularity and of focal or limited positivity by ICA in normal structures outside the neoplasm. Because immunocytochemical techniques cannot provide quantitative determinations but permit the evaluation of receptor distribution within the tumors, it is suggested that biochemical and histochemical methods should be employed together for steroid receptor determinations in breast cancer. This recommendation must be confirmed by clinical follow-up, particularly in discordant cases.

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Original Article

Immunocytochemical Analysis of Progesterone Receptors in Breast Cancer

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Breast cancer specimens from 116 patients were assayed for the presence of progesterone receptor (PR), with the use of a highly specific monoclonal antibody and the peroxidase–antiperoxidase technique on cryostat and permanent sections. Results were compared with those obtained by the conventional PR determination by dextran-coated charcoal (DCC) assay; they were in concordance in 90% of cryostat sections and 85% of paraffin-embedded tissue. The sensitivity and specificity of the PR immunocytochemical assay (PR-ICA) were 91% and 89% for frozen sections and 83% and 89% for permanent sections, respectively. The immunostained slides also were evaluated for several semi-quantitative features, including staining intensity, heterogeneity of staining, and the proportion of positive tumor cells. A statistically significant correlation was found between the percentage of tumor cells stained with the PR immunocytochemical tech-

nique and the PR–cytosol levels ($P < 0.05$). These results suggest that the PR-ICA is an effective tool in the evaluation of PR content in breast cancer and can be applied in paraffin as well as frozen sections. This technique provides excellent morphologic detail, as well as tissue localization for PR. It also offers an alternative for assessment of PR when fresh tissue is not available for conventional hormone receptor analysis. The immunocytochemical assay can be performed easily at community hospitals. Because it requires only a small amount of tissue, PR-ICA is an ideal method for analyzing specimens of insufficient size for the DCC assay. This technique also is suited to the evaluation of fine-needle aspiration biopsy specimens. (Key words: Progesterone receptor immunocytochemical assay; Dextran-coated charcoal assay) Am J Clin Pathol 1991;96:59–63

The presence of estrogen receptor (ER) in breast cancer not only predicts the response to hormone therapy, but is also a powerful independent prognostic factor.[1-3] Thus, treatment options often are based on identification of ER in tumor tissue. Recent studies also suggest that assessments of progesterone receptor (PR) are equally or more valuable than those of ER in predicting the disease-free interval in patients with breast cancer.[4-6] Therefore, measurement of ER and PR in tumor tissue is very important in patients with breast cancer.[7]

In addition to conventional biochemical assays, production by Greene and associates[8] and King and Greene[9] of a highly specific monoclonal antibody directed against

ER opened a new vista for immunocytochemical localization of ER protein. Since then, an immunoperoxidase technique using a specific monoclonal antibody to ER has been used repeatedly in immunocytochemical localization of ER protein in human breast tumors. This assay is very specific and sensitive.[10-12]

A monoclonal antibody against PR only recently has become available; therefore, unlike ER, it has not yet been studied extensively.[13] This prompted us to further evaluate immunocytochemical localization of PR in breast tumor tissue.

This article compares the immunocytochemical assay for PR with conventional biochemical analysis of receptor content in both fresh-frozen and formalin-fixed, paraffin-embedded breast tumor tissue. The biochemical assay is used as the standard, and the data are analyzed statistically to determine the sensitivity and specificity of the PR immunocytochemical assay (PR-ICA). A semiquantitative PR-ICA score is correlated with the PR level expressed in femtomoles/milligram protein.

MATERIALS AND METHODS

One hundred sixteen specimens from malignant breast tumors, received during a 32-month period, were used in

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this study. Breast tissue was received fresh for intraoperative consultation in the surgical pathology laboratory. Immediately after a diagnosis of cancer was established by frozen section or cytologic smears, a portion of the malignant tissue carefully was selected for the study and trimmed of adjacent fat and grossly benign breast parenchyma. Both the immunochemical and biochemical assays were performed by subdividing this single piece of tumor. Tissue for PR-ICA was frozen and stored at −70 °C until further processed. Tissue was stored no longer than six months before staining. The remaining half of the sample was quick frozen and submitted to a commercial laboratory (SmithKline BioScience) for the dextran-coated charcoal (DCC) biochemical assay. A positive biochemical assay was defined as a value greater than 10 fmol/mg of tissue protein.

The reagents used in the PR-ICA were donated by Abbott Laboratories (Chicago, IL) and used as suggested by the supplier. Cryostat frozen sections of breast tumors were fixed immediately in 3.7% formaldehyde–phosphate–buffered saline (PBS) for 15 minutes. Sections then were washed in PBS (0.01 mol/L, pH 7.4) for at least 5 minutes, immersed sequentially in cold methanol and acetone (both at −10 °C) for 3 minutes each, and then rinsed again in PBS. After this procedure, the sections were incubated successively in the following immunoreagents, with the appropriate PBS rinse: blocking reagent (normal goat serum) for 15 minutes, primary antibody (monoclonal antibody against PR, KD68) or negative control antibody for one hour, bridging antibody (goat antirat immunoglobulin) for one hour, and peroxidase–antiperoxidase (PAP) for one hour. After a final rinse in PBS, sections were incubated in the chromogen substrate solution (diaminobenzidine [DAB], hydrogen peroxide, PBS) for 8 minutes, rinsed in tap water, counterstained with dilute Harris hematoxylin, dehydrated, and coverslipped. The positive and negative control slides were treated similarly. PR-positive cells exhibited dark brown nuclear staining.

So that the potential value of PR-ICA could be assessed in formalin-fixed, paraffin-embedded tissue, the frozen tissue of the cases in this study in which PR analysis had been performed by biochemical DCC assay and PR-ICA were retrieved from our tumor bank and submitted for tissue processing. Paraffin tissue blocks were cut 4 μm thick, placed on Elmer's Glue®–coated slides, dried in an oven at 55 °C, deparaffinized in xylene, and hydrated and rinsed in 0.05 mol/L TRIS buffer (pH 7.4 at 25 °C). The sections then underwent PR-ICA as described previously. No enzyme pretreatment was necessary for PR immunostaining.

Two independent observers examined the test slides microscopically and recorded their findings. Results of immunostaining were expressed as the ratio of positive tumor cells–total tumor cells. The intensity of staining was evaluated on a scale ranging from negative through 3+. The strongest nuclear staining, exemplified by normal breast ductal cells, was defined as 3+; moderate brown staining as 2+; and faint and light nuclear staining as 1+. Negative cells were pale and did not stain. Trace reactions were considered negative. A tumor was considered "negative for PR" if all the neoplastic cells were negative; it was judged "positive for PR" if any neoplastic cells were read as 1+ or greater.

The data obtained were analyzed statistically for accuracy, sensitivity, and specificity. Accuracy reflects the degree the PR-ICA classification of cases agreed with classification as determined by the biochemical values. Sensitivity was expressed as the percentage of biochemically positive cases that were positive by the immunoperoxidase technique (percent true positive), and specificity was expressed as the percentage of biochemically negative cases that were negative by the immunoperoxidase technique (percent true negative).

For semiquantitative analysis, the overall staining intensity was multiplied by the fraction of positive cells; then this numeric value, designated the PR-ICA score, was compared with the PR content, expressed as femtomoles per milligram of protein. P values were obtained with a 2×2 contingency table and the chi-square test.

RESULTS

In this study, of the 116 patients with breast cancer, 93 had infiltrating duct cell carcinoma, 16 had lobular carcinoma, 4 had medullary carcinoma, and 3 had papillary carcinoma. The patient's ages ranged from 31 to 93 years.

Positive staining for PR was restricted to the nuclei of neoplastic cells (Fig. 1). Most of the specimens examined were composed of varying proportions of positive and negative tumor cells. The number of positive tumor cells in the cases studied ranged from 20% to more than 95%. There was no case with positive findings for PR in which less than 20% of tumor cells showed brown nuclear staining. So, in reality, cases that were considered positive were those with nuclear staining in ≥20% of tumor cells expressing staining intensity of at least 1+. No evidence of nuclear staining was seen in negative cases. Heterogeneity of staining was conspicuous. Overall, infiltrating duct cell carcinomas displayed greater PR heterogeneity compared with lobular carcinomas. Lobular carcinomas often showed diffuse and uniform patterns of nuclear staining.

The comparative results of this study are summarized in Tables 1–4. Table 1 shows the comparison of PR-ICA values on frozen sections with the DCC assay biochemical values. Forty-two patients (36%) had negative results by both methods, and 63 patients (54%) had positive results by both immunoperoxidase and biochemical determi-
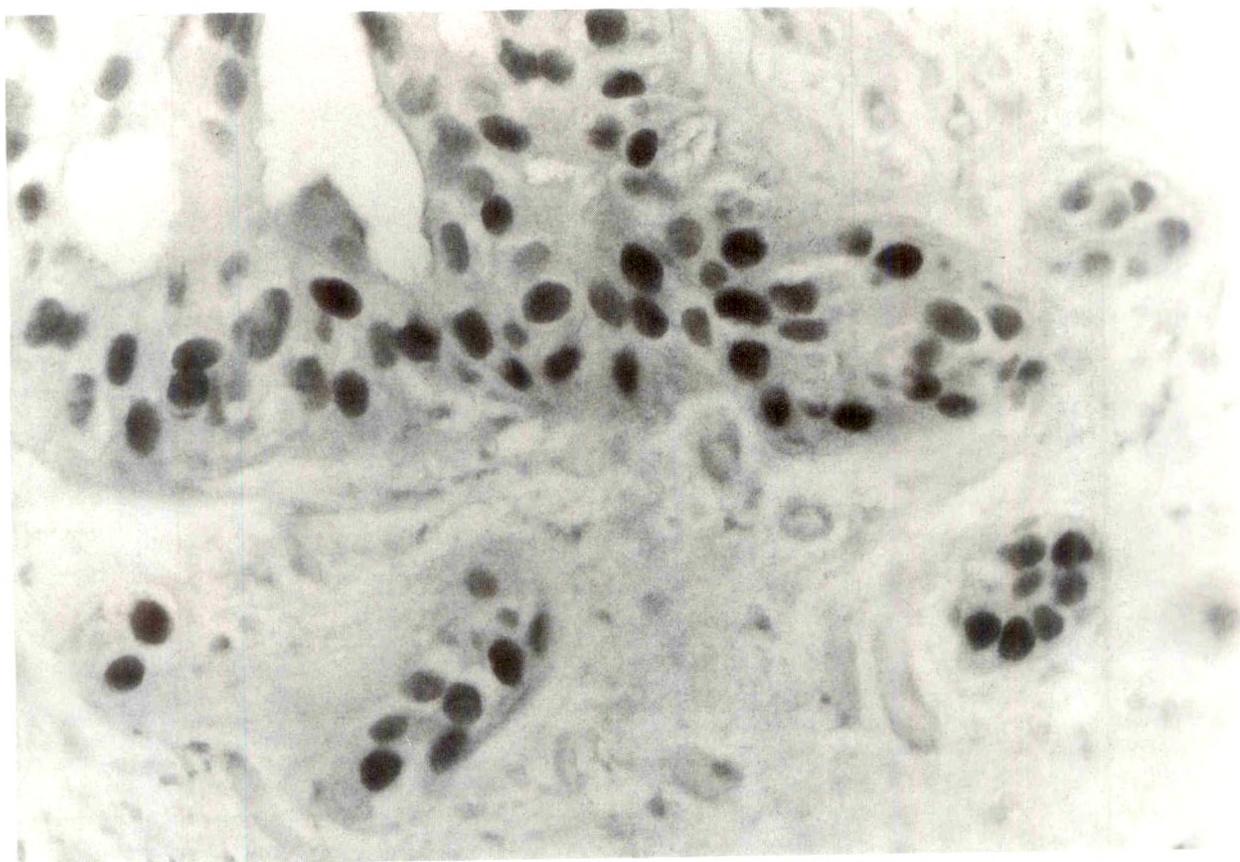
Fig. 1 (*upper*). Infiltrating, ductal carcinoma showing nuclear staining for progesterone receptor with monoclonal antibody. Antiprogesterone receptor protein immunostain (×400).

nations. Six biochemically positive specimens were not detected by the immunocytochemical technique. Five specimens with PR-negative results by the DCC assay had positive immunostaining. In comparison with the biochemical method, the immunocytochemical assay showed a sensitivity of 91% and specificity of 89%. Overall accuracy was 90% (Table 2).

With the use of paraffin-embedded tissue sections, there was an overall 85% concordance between PR-ICA and

DCC assay results, with a sensitivity of 83% and specificity of 89% (Tables 2 and 3). No statistically significant difference ($P > 0.05$) was found between the results of PR-ICA on frozen and formalin-fixed, paraffin-embedded tissue. As shown in Table 4, there is an overall 84% concordance between the PR-ICA results of frozen sections and formalin-processed tissue. Biochemical values for the so-called "false-negative" cases ranged from 41 to 220 fmol/mg protein. Cases that were biochemically negative

TABLE 1. COMPARISON OF DEXTRAN-COATED CHARCOAL ASSAY WITH PROGESTERONE RECEPTOR IMMUNOCYTOCHEMICAL ASSAY ON 116 FROZEN SECTIONS OBTAINED FROM PATIENTS WITH BREAST CANCER

DCCA* (fmol/mg)	No. of Cases	PR-ICA†	
		Negative	Positive
<10	47	42	5
>10	69	6	63

* Dextran coated charcoal assay in femtomole/milligram protein.
† Progesterone receptor immunocytochemical assay.

TABLE 2. DIAGNOSTIC ACCURACY, SENSITIVITY, AND SPECIFICITY OF PROGESTERONE RECEPTOR IMMUNOCYTOCHEMICAL ASSAY ON FROZEN AND PARAFFIN SECTIONS COMPARED WITH BIOCHEMICAL VALUES

PR-ICA*	Diagnostic Accuracy No. (%)		Sensitivity No. (%)		Specificity No. (%)	
Frozen sections	105/116	90%	63/69	91%	42/47	89%
Paraffin sections	99/116	85%	57/69	83%	42/47	89%

* Progesterone receptor immunocytochemical assay.

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TABLE 3. COMPARISON OF DEXTRAN-COATED CHARCOAL ASSAY WITH PROGESTERONE RECEPTOR ASSAY ON 116 PARAFFIN SECTIONS OBTAINED FROM PATIENTS WITH BREAST CANCER

DCCA* (fmol/mg)	No. of Cases	PR-ICA†	
		Negative	Positive
<10	47	42	5
>10	69	12	57

* Dextran-coated charcoal assay in femtomole/miligram protein.
† Progesterone receptor immunocytochemical assay.

(DCC assay level < 10 fmol/mg protein) and immunoperoxidase positive were labeled as having false-positive results; however, the alternative explanation, namely that the biochemical assay was inadequate because of sampling error, tumor heterogeneity, or loss of binding capacity during storage and transport, should be considered seriously. In these cases, PR-ICA could identify tumors with DCC assay levels ranging from 7 to 9 fmol/mg cytosolic protein. These were cases with 35–60% of tumor cells showing 1+ to 2+ brown nuclear staining.

The relationship between PR results obtained by the DCC assay and the semiquantitative PR-ICA results is shown in Figure 2. A statistically significant correlation ($P < 0.05$) was found between the intensity and heterogeneity by immunocytochemical staining and biochemically determined PR levels.

DISCUSSION

The demonstration of ERs in human breast cancer by Jensen and associates[14] in 1971 has provided important information about the role of estrogen in maintaining the growth of breast cancer. Jensen and colleagues were also the first to suggest that the presence of ERs in breast cancer was correlated with response to endocrine therapy. This observation has been confirmed repeatedly by several other investigators.[3,15] Despite this knowledge, more than 40% of ER-positive tumors do not respond to endocrine therapy.[3] Several strategies have been developed to increase the accuracy of selecting patients for endocrine therapy. Among the most promising are the simultaneous

TABLE 4. COMPARISON OF PROGESTERONE RECEPTOR IMMUNOCYTOCHEMICAL ASSAY ON PARAFFIN AND FROZEN SECTIONS

Frozen Sections	No. of Cases	Paraffin Sections	
		Negative	Positive
Negative	48	42	6
Positive	68	12	56

Concordance 98 of 116 (84%).
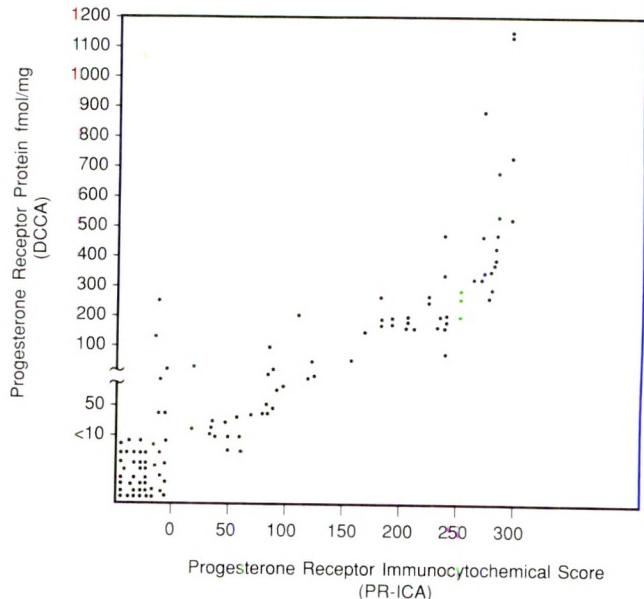
FIG. 2 (*lower*). Comparison of dextran-coated charcoal and progesterone receptor immunocytochemical assays on frozen tissue by 116 patients with breast cancer.

determination of PR[16] and the development of more practical techniques for evaluating ER and PR proteins.[8,9]

Recent data from several studies indicate that PR, as an end product of estrogen action, is an even better indicator of favorable prognosis and endocrine responsiveness than ER in breast cancer.[4] Most studies on PR content of breast tumor tissues have relied on steroid-binding assays performed with radiolabeled progestins. These assays, involving homogenization of the tissue, do not localize the receptor to specific cells. Thus, no information is available about the intensity, proportion, and distribution of receptor-containing cells.

If receptor heterogeneity is an indicator of cancers that are less likely to respond to endocrine therapy because of the presence of autonomous ER- and PR-negative tumor cells, the ability to identify such cells may be important in determining the appropriate therapy and the course of the disease.[17] In addition, current biochemical assays require from 0.5 to 1 g of tissue and are not suitable for assessment of PR content of small tumors, metastatic effusions, and needle biopsy specimens. Furthermore, conventional biochemical assays for measuring PRs require expensive ultracentrifugation equipment, scintillation counters, and radiolabeled chemicals. Consequently, these methods are used only in commercial laboratories and large medical centers.

In our hands, the PR content identified by nuclear localization by anti-PR monoclonal antibody in breast tumor tissue compared favorably with the PR content measured biochemically in these lesions. A high degree of

concordance was found between DCC assay biochemical values and the results of the immunoperoxidase technique.

The discrepancies between the results of the PR-ICA and the DCC assay may be explained on the basis of sampling, tumor heterogeneity, and loss of immunoreactivity or binding capacity during storage and transport. The presence of intermixed benign epithelial components, which frequently may be included in the biochemical assays, is another major factor contributing to "false-negative" results by the immunostaining technique. Review of our false-negative immunocytochemical cases revealed the presence of PR-negative tumor cells surrounded by brown nuclear staining in adjacent nonneoplastic epithelial cells.

With the use of a simple score based on heterogeneity and staining intensity for semiquantitation of PR-ICA, it was possible to show a statistically significant correlation between PR levels assayed biochemically and those estimated by PR-ICA.

In the current investigation, we also have demonstrated that PR-ICA can be applied reliably in the detection of PR in formalin-fixed, paraffin-embedded tissue. This will provide a suitable alternative when fresh tumor tissue is not available or suitable for biochemical assay or conventional immunocytochemical assay. This technique also has the potential to be used in retrospective hormone receptor studies of patients with breast cancer with known clinical outcome.

In the current study, the immunostaining results in both paraffin-embedded and frozen sections correlate well with each other and with the values obtained by the DCC assay. However, as shown in Table 2, although this technique is highly specific, PR-ICA is less sensitive in permanent sections than in frozen sections. The greater number of false-negative results in permanent sections, responsible for this relative decrease in sensitivity, may result from a loss of immunoreactivity during fixation and tissue processing rather than limitation in the sensitivity of this immunostaining technique. Therefore, negative immunostaining results for PR on permanent sections should be interpreted with caution. A negative immunostaining result for PR on tumor tissue is definitely valid when evidence of PR expression is seen in benign epithelial components of breast tissue in the same block of tissue examined.

Overall, this study shows that an immunocytochemical technique using a specific monoclonal antibody against PR (KD68) can detect PR in breast tumor cells. Further-

more, a semiquantitative estimate of PR content is possible. Additional investigation is warranted, especially in consideration of the potential value in assessment of PR status in fine-needle aspirates and malignant effusions. We are extending our investigation into these areas and evaluating the use of computer-assisted image analysis for quantitation of hormone receptors.

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Automated Procedure for Dewaxing and Rehydration of Paraffin-Embedded Tissue Sections for DNA Flow Cytometric Analysis of Breast Tumors

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Flow cytometric DNA analysis of paraffin-embedded tumors is an important diagnostic and prognostic tool in clinical pathology. The technique is limited, however, by the time-consuming multistep procedure for dewaxing and rehydrating tissue. The authors developed an automated procedure to complete the dewaxing and rehydration of tissue using a routine histologic tissue processor with a 24-hour timer. This technique provided excellent tissue recovery and reproducible DNA histograms comparable to those obtained by manual methods. Subsequently, the authors analyzed the DNA content of 93 paraffin-embedded breast cancer tissues. The automation of a significant portion of the routine processing required for paraffin-embedded tissue makes cytometric DNA analysis a more practical procedure in the laboratory. (Key words: Flow cytometry; Paraffin-embedded tissue; Tissue processing; DNA analysis) Am J Clin Pathol 1991; 96: 64–69

Flow cytometric analysis of DNA content in single cells rapidly and accurately determines the cell cycle distribution and ploidy. Hedley and associates[1] recently developed a technique that allows the flow cytometric quantitation of cellular DNA content in previously paraffin-embedded pathologic material. This method has permitted numerous retrospective studies evaluating the presence or absence of DNA-aneuploid stemlines and cell cycle distribution. The study of paraffin-embedded tissues still has many limitations: (1) the time-consuming and laborious multistep procedure for dewaxing and rehydrating tissue; (2) the inability to use routine internal standards in formalin-fixed tissue; (3) the effect of tissue fixation on ploidy analysis; and (4) relatively poor resolution compared with that of fresh material. To improve this difficult technique, we developed an automated procedure to complete the dewaxing and rehydration of tissue while it is in a single processing and embedding cassette.

In this article, we report the efficiency of this automated technique in a study of paraffin-embedded breast cancer tissues.

MATERIALS AND METHODS

Tissue Preparation

Ninety-three samples from 69 patients were analyzed for cellular DNA content. The patients' slides were reviewed, and, where possible, at least two representative blocks of normal and malignant paraffin-embedded tissues were selected for DNA flow cytometry. The appropriate blocks were retrieved, and three 50-μm sections were cut from each of them. Occasionally, more sections were required if the tissue block was small. To determine the tissue representation and confirm the original diagnosis, a routine 5-μm section also was cut after each series of 50-μm sections and stained with hematoxylin and eosin.

Before all 93 samples were analyzed, the manual and automated dewaxing and rehydrating methods were compared side by side, using an identical set of samples (five breast carcinomas, one lymph node), to determine the reproducibility of the two techniques. Briefly, for the manual method, a modification of the procedure of Hedley and associates[1] was used. Tissue sections were placed

in glass centrifuge tubes and dewaxed with three changes of xylene (3 mL for 10 minutes at room temperature) and rehydrated in a series of ethyl alcohol strengths (3 mL of 100, 95, 70, and 50% for 10 minutes each) at room temperature. The tissue then was washed twice in distilled water. In the automated method, each set of sections was placed in an aspiration bag (Shandon Inc., Pittsburgh, PA) and enclosed in a processing and embedding cassette. The cassettes were placed in a metal cassette carrier that holds as many as 30 cassettes.

The tissue was dewaxed with three changes of xylene and rehydrated in the same series of graded ethanols used in the manual method, followed by two final washes in distilled water. The solutions were kept in 1-L volumetric beakers and arranged sequentially on a tissue-processing machine (Sakura Fine Technical Company, Ltd., Tokyo, Japan) (Fig. 1). The entire multistep dewaxing and rehydrating procedure was performed automatically on the tissue processor, as the cassette carrier rotated sequentially from one solution to the next.

The processor was equipped with a 24-hour automatic timer, yielding a minimum incubation interval of 30 minutes in each solution. To initiate automated dewaxing and rehydration, the samples first were placed in a dry beaker with the timer set to rotate the cassette carrier into the first dewaxing solution within 15 minutes. Once the carrier passed through all the required solutions, it remained in the last beaker of distilled water until it was removed from the processor. Uniform mixing was ensured throughout the procedure, as the tissue processor continuously rotated the cassette carrier from left to right. After the final wash in distilled water, the cassette carrier was removed and each cassette was opened. The tissue was removed from the aspiration bags with fine-tip forceps, placed in 1.5-mL polypropylene Eppendorf tubes (Brinkmann Instrument Company, Westbury, NY), and minced with straight scissors while immersed in distilled water. Tissue that was dewaxed and rehydrated manually was minced and digested enzymatically in the same manner as that which was prepared automatically.
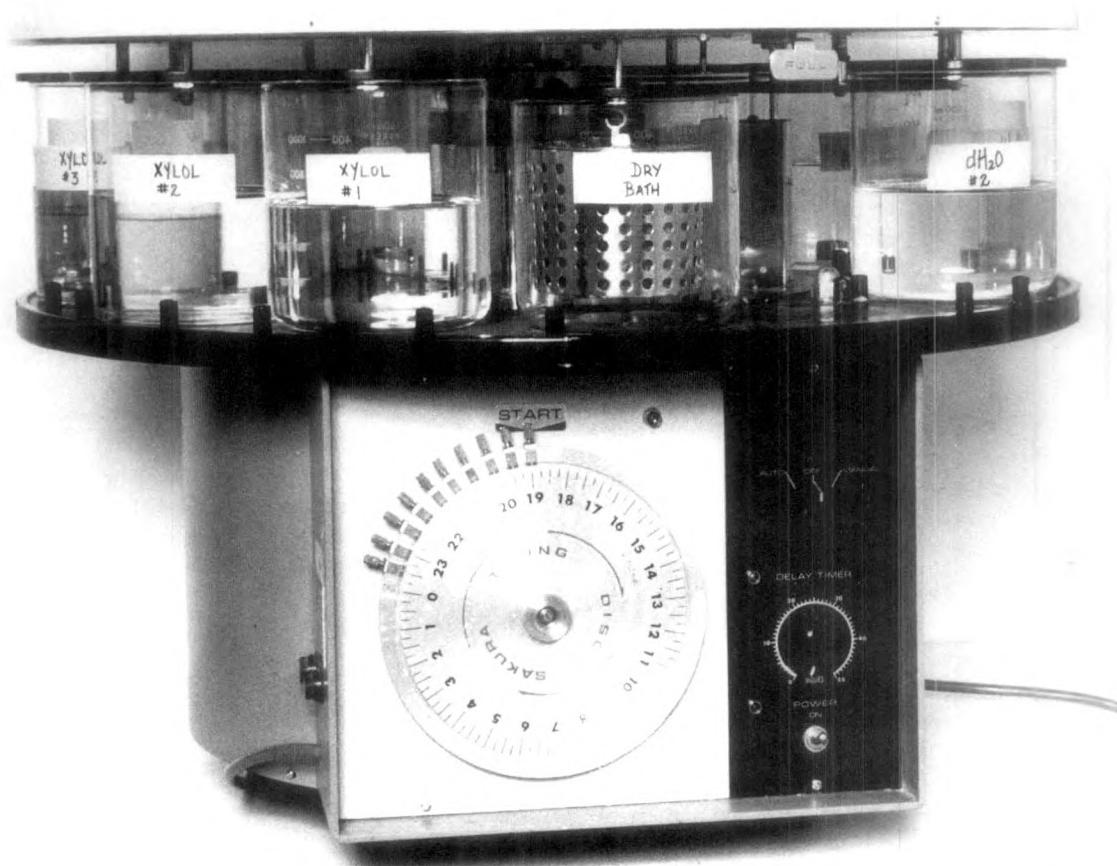
FIG. 1. Routine histologic tissue processor equipped with a 24-hour timer to automatically commence dewaxing and rehydration of paraffin-embedded tissue. A metal cassette carrier is used to hold multiple processing and embedding cassettes. Volumetric beakers (1,000 mL) contain the solutions through which the cassette carrier sequentially rotates.

The tissue was transferred to 15-mL tubes, centrifuged (all centrifugations were for 10 minutes at $500 \times g$, 4 °C), and resuspended in 1 mL of 0.5% (weight/volume [w/v]) pepsin (Sigma Chemical Company, St. Louis, MO) in 0.9% (w/v) NaCl (adjusted to pH 1.5 with 2 N HCl) containing 3% (w/v) polyethylene glycol (PEG) 6000. The tubes were placed in a 37 °C water bath for 30 minutes, with brief vortexing at 5-minute intervals. The resulting nuclei then were filtered through a 53-μm nylon mesh (Spectrum Medical Industries, Inc., Los Angeles, CA) into a clean 15-mL centrifuge tube, and the pH was neutralized by increasing the volume to 5 mL with cold 25 mmol/L HEPES–Hank's balanced salt solution (HBSS) containing 3% (w/v) PEG 8000 and 0.02% (w/v) azide. The suspensions were centrifuged, and the nuclear pellets were resuspended in 2 mL HEPES–HBSS. An aliquot was removed for determination of nuclear yield, to ensure that recovery was no greater than 2×10^6 cells/mm^3. When nuclear counts exceeded this figure, an appropriate aliquot was removed for DNA staining. It was found that samples could be stored in this solution for several days under refrigeration at 4 °C.

DNA Staining

The suspensions were centrifuged and resuspended in 0.5% (w/v) paraformaldehyde in phosphate-buffered saline (PBS), at a concentration of 1–2×10^6 nuclei/mL, for 10 minutes at 4 °C. After another centrifugation, 1 mL of cold 0.1% (v/v) Triton X-100® (Sigma Chemical Company, St. Louis, MO) in PBS was added to the samples and left to incubate on ice for 3 minutes, to render the nuclei permeable to the staining fluorochrome. Again, the nuclei were centrifuged and resuspended in RNAse (0.180 units/L in PBS) for 30 minutes at 37 °C to remove residual RNA. After centrifugation, the nuclei were stained with 50 μg/mL propidium iodide (PI) (Sigma) in PBS. All PI-stained samples incubated for at least 30 minutes before flow cytometric analysis.

Flow Cytometric Analysis

The stained nuclear suspensions were analyzed using a FACScan® flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA), with a 488-nm argon laser as an excitation source. Instrument calibration and performance were monitored with CaliBRITE® beads and the Autocomp® software designed by Becton Dickinson Immunocytometry Systems. Information regarding instrument linearity and resolution was obtained by analyzing ethanol-fixed chicken erythrocyte nuclei. Before flow analysis, each sample was passed several times through a 25-gauge needle with a syringe and filtered through a 53-μm nylon mesh filter. At least 20,000 events were measured per sample, and low flow rates (12 μL/

minute) were used to maximize resolution. The DNA index (DI) and cell cycle distribution were determined with the CELLFIT® software developed by Becton Dickinson.

RESULTS

After comparison of the results of the same set of samples (five carcinomas, one lymph node) that were prepared manually and automatically, no significant differences were identified. The coefficients of variance (CVs) were comparable in both sets, no loss of aneuploid populations was detected, and the DNA staining patterns were unremarkable as judged by the reproducible histograms (Fig. 2).

After the two methods were compared side by side, 93 specimens from 69 node-negative breast cancer patients were prepared automatically and evaluated. The tissues included 24 uninvolved lymph nodes and 69 breast carcinomas of various types. Only one tumor sample was rejected because of significant baseline debris and an uninterpretable histogram. Of the remaining 68 carcinomas, the quality of the DNA histograms was acceptable in 90% (61 of 68) of the samples analyzed. Although appearing diploid, seven tumor samples had significantly greater and unacceptable CVs (8.3–16.2). (In this study, the CV of the G_0/G_1 peak width of all tumors had to be less than 5.0 for the specimen to be considered interpretable for ploidy status).

Although no external standard can be used to identify the diploid population positively in paraffin-embedded material, 24 normal uninvolved lymph nodes were available as diploid standards. In the tumor samples in which an external diploid standard was available, most (20 of 24) G_0/G_1 peaks coincided with the peak that was defined as diploid in the lymph nodes. Four tumors had single G_0/G_1 peaks that did not coincide with the defined normal populations.

The DI was determined according to a recently suggested convention on nomenclature for DNA cytometry.[2] Therefore, DI was defined as the ratio of the mode of the relative DNA content of the G_0/G_1 cells of the sample divided by the mode of the relative DNA measurement of the diploid G_0/G_1 reference cells. By definition, cells with a normal diploid karyotype have a DI of 1.0. A DNA histogram was said to contain a DNA aneuploid population when at least two separate G_0/G_1 peaks were demonstrated. For the paraffin-embedded samples, the first peak in the histogram was considered as a normal diploid standard. Of the 61 tumor samples examined, 25 (41%) were diploid and 36 (59%) were aneuploid. The distribution of DIs ranged from 1.08 to 2.17. The mean CVs for the G_0/G_1 peak of the diploid and aneuploid tumors were 4.54 and 4.18, respectively. Representative DNA
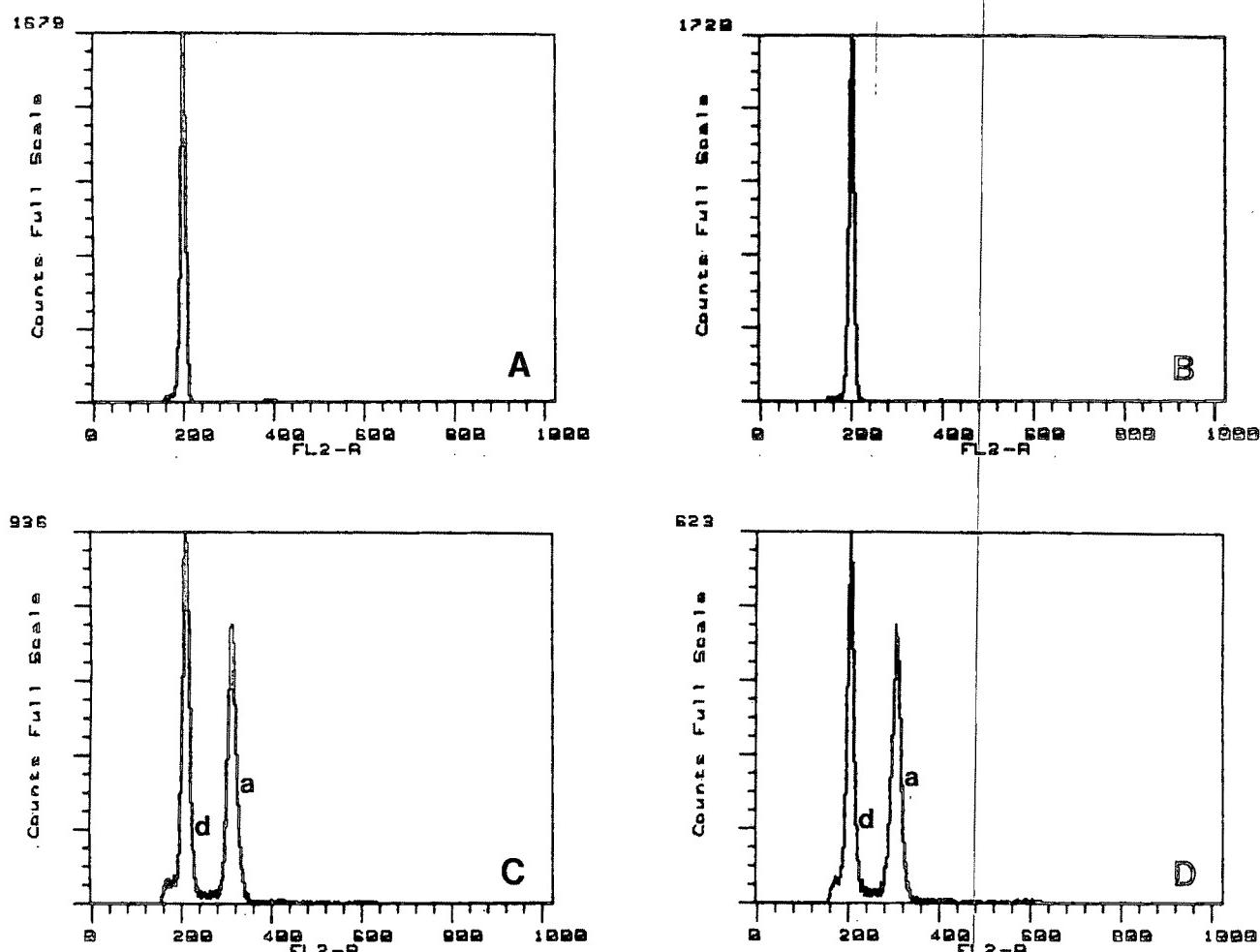
FIG. 2. Side-by-side comparison of the DNA histograms from the same set of samples prepared manually and automatically. The number of particles is given on the vertical axis and DNA content (channel numbers) on the horizontal axis. Coefficient of variance (CV) of the G_0/G_1 peak of the lymph node prepared (*A*) manually and (*B*) automatically was 2.8 and 2.9, respectively. CVs of the diploid populations of the tumor prepared (*C*) manually and (*D*) automatically were 3.6 and 3.3, respectively. Under the same conditions, the CVs of the aneuploid G_0/G_1 peaks were 2.9 and 3.4, respectively. DNA indices of the tumor were similar: manual DI = 1.49 and automated DI = 1.48.

histograms from paraffin-embedded tissue that was dewaxed and rehydrated automatically are shown in Figure 3.

DISCUSSION

The development of a method for flow cytometric DNA analysis of routinely prepared paraffin-embedded tissue by Hedley and colleagues[1] has permitted numerous retrospective clinical studies. The method also allows rare, but clinically important, tumors to be studied because samples can be pooled from several institutions. Moreover, it permits assessments of DNA content as an independent prognostic variable in patients whose clinical outcome is known.

The technique is long and tedious, and we have modified it to reduce the laborious and time-consuming mul-

tistep procedure for dewaxing and rehydrating tissue. With most techniques, this step is performed with the tissue in glass centrifuge tubes, with serial decantation or aspiration. Sickle-Santanello and associates[3] demonstrated the use of a specially designed container to hold multiple processing and embedding cassettes, to minimize the time required for individual samples to be dewaxed and rehydrated.

Herein, we report a technical improvement that automates this entire step by using a routine histologic tissue processor. This method reduces the amount of technologist time required to perform this laborious step because the timer on the processor can be set to start the automatic dewaxing and rehydration process. When the process is started after laboratory hours, samples can dewax and rehydrate overnight and be available early the next day for enzymatic digestion. Although the tissue samples incubate in each solution for 30 minutes, rather than the
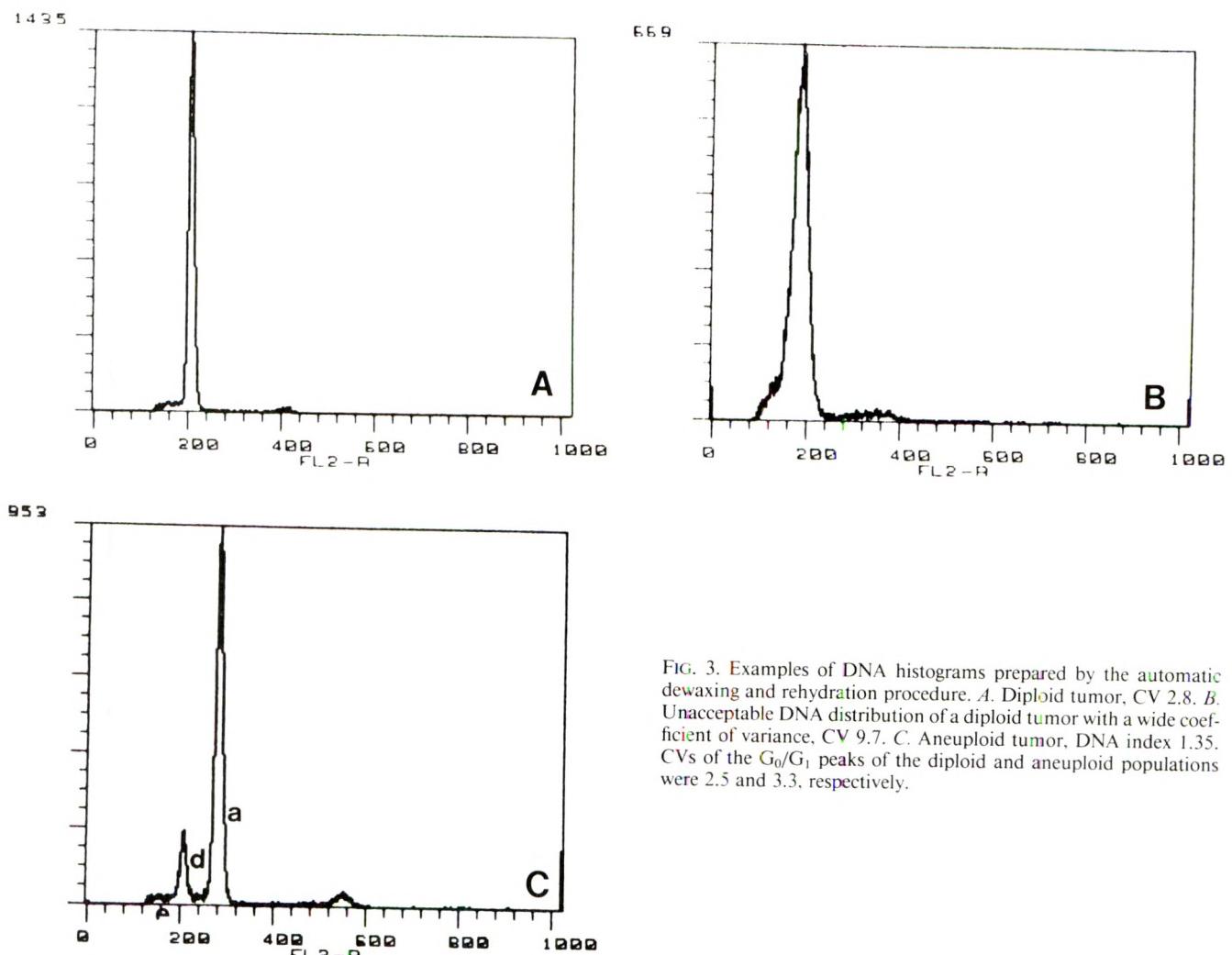
FIG. 3. Examples of DNA histograms prepared by the automatic dewaxing and rehydration procedure. *A.* Diploid tumor, CV 2.8. *B.* Unacceptable DNA distribution of a diploid tumor with a wide coefficient of variance, CV 9.7. *C.* Aneuploid tumor, DNA index 1.35. CVs of the G_0/G_1 peaks of the diploid and aneuploid populations were 2.5 and 3.3, respectively.

previously suggested 10 minutes, this has no effect on the reproducibility or the quality of DNA staining. Actually, the time of the automated procedure is lengthened, but, more importantly, the technique can be performed by a machine, freeing the technologist for more productive work.

We found that tissue recovery was excellent, and the performance of all steps while the tissue was in an aspiration bag enclosed in a single cassette minimized the chance of losing or fragmenting the tissue. In addition, because more samples can be batched during a single run, the efficiency of this technique is increased; the cassette carrier can hold as many as 30 cassettes.

Ninety-three samples (69 tumors, 24 uninvolved lymph nodes) were prepared automatically after a side-by-side comparison of the two methods. There were no significant differences in the same set of samples. The resolution (CV), recovery of aneuploid populations, and DNA staining were all comparable. The 59% frequency of DNA

aneuploidy for the breast carcinomas in this study agrees with a number of previous reports.[4-9] In 24 of the 69 patients with breast cancer, an external diploid standard was available from samples of normal lymph node to help characterize ploidy more accurately. In four tumor samples, the G_0/G_1 peak did not coincide with the external diploid cells, possibly because of differences in fixation that we cannot control with the use of archival specimens. Seven tumor samples with higher CVs (8.3–16.2) were excluded from our analysis because of the risk of overlooking near-diploid tumors. Hedley and colleagues suggested that a greater proportion of paraffin-embedded tumors have a DI of 1.0, but this is associated with fewer near-diploid tumors (*i.e.,* DI >1.0–1.1) when DNA analysis is performed on both fresh and paraffin-embedded material.[10] Hence, our percentage of diploid tumors may not be statistically significant because the full extent to which an increasing CV leads to loss of near-diploid G_0/G_1 peaks remains uncertain.

Dewaxing and Rehydration of Tissue

In conclusion, the technical improvement we have described in this study yields reproducible results and is more efficient than previously suggested methods. It opens the way for other technical advances to further improve the paraffin-embedded technique for DNA flow cytometric analysis and to reduce its current limitations.

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Correlation Between Visual Clues, Objective Architectural Features, and Interobserver Agreement in Prostate Cancer

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Three pathologists evaluated a number of designated architectural features to assign grades to 41 cases of well- to moderately differentiated adenocarcinoma, and their opinions were compared. The consensus opinion was obtained and evaluated against objective measurements of glandular architecture that were obtained by morphometric techniques. The observers agreed on gland size, gland uniformity, and the number of glands per field in only 49%, 31%, and 39% of cases, respectively. There were significant differences in the Gleason grades assigned by observers. Paired matching of individual Gleason grades showed agreement among observers in 44% (18 of 41), 56% (23 of 41), and 75% (31 of 41) of cases, respectively. This level of interobserver disagreement occurred even though cases with predominant patterns were selected carefully and those with variable patterns were excluded. A direct relationship appears to exist between increasing Gleason grade and increasing glandular variability, and there is an inverse relationship between Gleason grade, gland lumen area, and the number of glandular nuclei, as assessed by a group of pathologists. (Key words: Grading; Prostate cancer; Morphometry; Architectural features; Gleason grade) Am J Clin Pathol 1991;96: 70–75

It is often difficult to grade neoplastic lesions subjectively, and the process is prone to significant interobserver variation. Although poorly differentiated carcinoma is easy to identify, the separation of moderately from well-differentiated lesions is often challenging, even for the experienced observer.

This study explored the use of objective measurements to improve the subjective decision-making process involved in categorizing Gleason grade 2 and 3 (*i.e.*, well- and moderately differentiated) prostatic adenocarcinomas.

To achieve this goal, the subjective opinions of three pathologists were compared, for which they used a number of designated architectural features to assign a grade. The consensus opinion was obtained and evaluated against objective measurements of glandular architecture that were obtained by morphometric techniques.

MATERIALS AND METHODS

Forty-one patients with adenocarcinoma of the prostate were studied. Fourteen specimens were obtained by needle biopsy, 12 by transurethral resection of the prostate (TURP), and 15 by total surgical prostatectomy. Formalin-fixed, paraffin-embedded prostate tissue was cut in 4-μm sections and stained with hematoxylin and eosin. Separate sections were stained with the Feulgen method for objective measurements.

Subjective Evaluation

Needle biopsy specimens were evaluated as a whole. A single representative section from prostatectomy and TURP specimens was chosen for evaluation. Cases of predominantly poorly differentiated adenocarcinoma

TABLE 1. GLEASON SCORE (WHOLE SLIDE) PATHOLOGISTS *VERSUS* CONSENSUS

| Pathologist Number | Perfect Agreement | Disagreement | | Total |
		±1*	±2*	
1	30/41 (73%)	7/41 (17%)	4/41 (9%)	41
2	28/41 (68%)	10/41 (24%)	3/41 (7%)	41
3	29/41 (70%)	12/41 (29%)	0/41 (0%)	41

* Grading score.

(Gleason grades 4 and 5) were excluded from the study. The participants in the evaluation were asked to assess the predominant architectural patterns of any given tumor. To facilitate this process, the areas of interest on the slides were circled with a felt-tip pen. The minor tumor architectural pattern was considered to be outside the circled areas.

To determine and evaluate the mechanisms by which pathologists assign a tumor grade, the group developed a set of visual clues. The final version contained 20 visual diagnostic clues, but only 5 features related to tissue architecture were included in the current study:

I. Separate Gland Type
  A. Predominantly closely packed
  B. Predominantly loosely packed
II. Fused Gland Type
  A. Cribriform
  B. Papillary
III. Gland Size (predominant)
  A. Very small
  B. Small
  C. Intermediate
IV. Gland Uniformity
  A. Uniform
  B. Mildly variable
  C. Variable
V. Gland Density
  A. Abundant
  B. Moderate
  C. Scanty

After assessing the five architectural visual clues independently, each pathologist selected a primary Gleason grade for the most prominent pattern within the designated area, as well as a Gleason score for the entire slide.

The histologic criteria used in the Gleason grading system are well known and were agreed upon before this study. In particular, Gleason grade 2 adenocarcinomas consist of single, separate, loosely aggregated, uniform glands. Gleason grade 3 tumors, on the other hand, are composed of single, separate, variably sized glands, in varying distribution, or of sharply circumscribed, papillary, and cribriform masses.[1,2] Summation Gleason scores followed the form a + b = c, where a equals the Gleason grade of the most prominent pattern; b equals the Gleason grade of the second most prominent pattern, and c equals the numeric sum of a + b (grading score). For example, visual diagnostic clues regarded as characteristic of Gleason grade 2 tumors included small, predominantly packed, slightly irregular glands of moderate to high density. Gleason grade 3 lesions showed visual diagnostic clues that were characterized by separate, variably sized, loosely packed glands, of moderate to low density. Papillary and cribriform fused gland types also were included in this category.

Subsequently, the three pathologists met with a moderator, who led the discussion of the cases and obtained a consensus diagnosis for each visual clue, Gleason grade, and total score in all cases.

Architectural Measurements

For architectural measurements, a previously described system[3] was used, which allows the pathologist to interactively mark any point in a static field displayed on a television screen, by moving a computer cursor. After preliminary tests, a decision was made to define the area of the gland by marking the centers of the glandular nuclei lining each lumen and connecting them. The remaining isolated tumor cell nuclei in the field were marked separately.

Because of the magnification used for architectural measurements (40 × and 6.3 trinocular set), only the

TABLE 2. GLEASON SCORE (WHOLE SLIDE) INTEROBSERVER AGREEMENT

| Pathologist 1 versus 2 | | | Pathologist 2 versus 3 | | | Pathologist 1 versus 3 | | |
| Agreement | Disagreement | | Agreement | Disagreement | | Agreement | Disagreement | |
	±1*	±2*		±1*	±2*		±1*	±2*
19/41 (46%)	13/41 (31%)	9/41 (22%)	20/41 (48%)	18/41 (44%)	3/41 (7%)	23/41 (56%)	15/41 (36%)	2/41 (5%)

* Grading score.

TABLE 3. PRIMARY GLEASON GRADE (INSIDE CIRCLE) PATHOLOGISTS *VERSUS* CONSENSUS

Pathologist Number	Perfect Agreement	Disagreement		Total
		±1 grade	±2 grade	
1	32/41 (78%)	8/41 (19%)	0	41
2	24/41 (58%)	17/41 (41%)	0	41
3	36/41 (88%)	5/41 (12%)	0	41

boundaries of the smaller glands inside larger glandular structures (such as in the cribriform pattern) were marked. Architectural measurements were taken in five fields from each case, within the previously circled area, enabling an average of 2,000 nuclei to be marked per case. The five selected fields were considered to be the most representative of the predominant glandular differentiation pattern for each case. In all instances, glands that were artifactually distorted or fragmented were excluded from the study.

RESULTS

In comparing the Gleason score for the entire slide that was assigned by each pathologist with the consensus score, agreement was achieved in 68–73% of the cases. Disagreement by one grade or more occurred in 7–29% of cases (Table 1). When the Gleason scores determined by two pathologists were compared, agreement was achieved in 46–56% of the cases, and disagreement by one grade or more varied from 5 to 44% (Table 2). For the Gleason grade of the predominant pattern (inside the circled area), agreement between the scores by each pathologist and the consensus score was obtained in 58–88% of the cases, with disagreement of one grade varying from 12 to 41% (Table 3). Comparison of two pathologists' opinions on the Gleason grade of the predominant pattern showed agreement in 44–75% of cases, and disagreement by one grade in 22–56% (Table 4). The level of agreement among the three pathologists for each of three visual clues showed poor agreement, ranging from 31 to 49% of cases. For cases in which an opinion differed by only one degree from the other opinions, the level of agreement varied from 44 to 58% (Table 5).

Despite significant interobserver variation, as described above, a relationship existed between the consensus Gleason grade and the objective architectural measurements obtained with the use of the cell image analysis system. Variability in gland size, as measured by variance of the mean nuclear distance to the gland center against variance of mean gland size, showed a continuous linear relationship between increasing variability in glandular architecture and a change from Gleason grade 2 to grade 3 carcinoma (Fig. 1). Likewise, a less clearly defined linear relationship was demonstrated between increasing glandular size and change in grade from Gleason grade 3 to grade 2 carcinoma (Fig. 2).

Each feature was analyzed statistically, and Table 6 shows their discriminating potential in moderately and well-differentiated tumor groups.

DISCUSSION

Most of the current systems used to grade prostatic adenocarcinoma are based on the subjective evaluation of morphologic features[1,2,4–7] that are subject to degrees of interobserver diagnostic variation.[8–10] This study assessed the variation in the diagnostic interpretation by three different pathologists of five tissue architectural features and compared these interpretations with the results obtained from the objective measurement of the same features.

In the current study, with a larger and more random group of cases than that of a previous analysis,[3] we evaluated the factors involved in diagnostic decision-making. It is important to understand this process, so that a grading system based on objective measurements can be developed.

The most significant finding was the extent of interobserver disagreement in the evaluation of visual clues. There was agreement among observers on gland size, gland uniformity, and the number of glands per field in only 49%, 31%, and 39% of cases, respectively (Table 5). This level of interobserver variation occurred even though tumors with predominant patterns were selected carefully and others with variable patterns were excluded. Subjective evaluation of visual clues seems to involve an un-

TABLE 4. PRIMARY GLEASON GRADE (INSIDE CIRCLE) INTEROBSERVER AGREEMENT

Pathologist 1 versus 2		Pathologist 2 versus 3		Pathologist 1 versus 3	
	Disagreement		Disagreement		Disagreement
Agreement	±1 Grade	Agreement	±1 Grade	Agreement	±1 Grade
18/41 (44%)	23/41 (56%)	23/41 (56%)	18/41 (43%)	31/41 (75%)	9/41 (22%)

TABLE 5. VISUAL CLUES INTEROBSERVER AGREEMENT

Gland Size	Gland Uniformity	Glands per Field
Perfect agreement (3 pathologists) 20/41 (49%)	Perfect agreement (3 pathologists) 13/41 (31%)	Perfect agreement (3 pathologists) 16/41 (39%)
Disagreement by one degree 18/41 (44%)	Disagreement by one degree 23/41 (56%)	Disagreement by one degree 24/41 (58%)

avoidable degree of variation in the interpretation of corresponding criteria. This is a dynamic process, because experience acquired in evaluating earlier cases exerts an influence on subsequent interpretations.

Another important point is that pathologists are not accustomed to analyzing each visual clue separately, without being influenced by their impression of the final

diagnosis. For example, a case might be interpreted as consisting of small glands, but only because the glands were smaller than those expected in a Gleason grade 3 tumor. However, if the tumor were considered by subjective evaluation to be in the Gleason grade 2 group, it might be interpreted as having intermediate-sized glands, even though there were as many large glands as medium-sized glands (Fig. 3). In such cases, a separate architectural or nuclear feature, such as the presence of nucleoli, may have influenced the pathologist's decision. In some cases, then, the pathologist may have assigned a Gleason grade based on his impression or *gestalt* of the pattern and then assigned values to each criterion to fit what would be expected of the assigned grade.

Another possible source of misinterpretation was the influence of extremes of pattern within a tumor. For example, a neoplasm composed mainly of small glands but also containing a few very large glands could have been interpreted as having intermediate-sized glands (Fig. 4).

It is not surprising, therefore, that significant differences occurred among observers in their selection of a Gleason grade. Paired matching of individual Gleason grades (major pattern) showed agreement among observers in 44% (18 of 41), 56% (23 of 41), and 75% (31 of 41) of cases, respectively. This interobserver disagreement was similar to that reported by others.[9,10]

A trend toward agreement between the results of the objective measurements and the *consensus* evaluation of Gleason grade (major pattern) by the pathologists was evident, as shown by Figures 1 and 2. This was less clear when the results of the objective measurements were matched against the individual opinion on the corresponding visual clue. The most important reason for this discrepancy was a lack of uniformity in the interpretation and application of criteria. In this regard, differences in the interpretation of the number of glands per field and the glandular size were particularly difficult (Fig. 5). Finally, in some cases, two distinct patterns were seen in approximately equal proportions, complicating the choice of a primary Gleason grade. It was not always possible to exclude one of the patterns from the study because patterns often were admixed closely (Fig. 4). In some cases, differences of opinion on Gleason grade for the entire
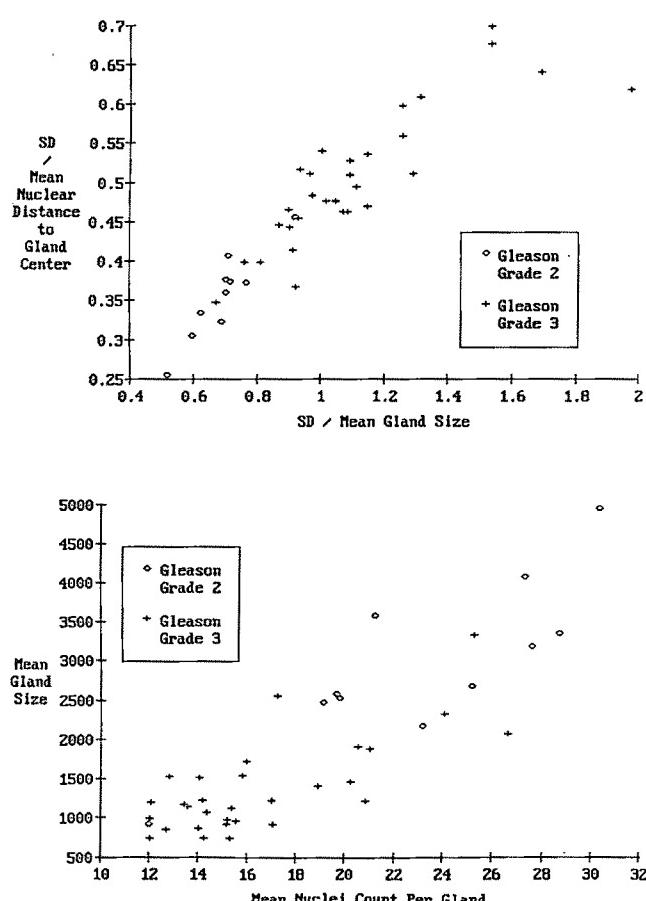
FIG. 1 (*upper*). Scatterplot of the case-wise standard deviation of mean nuclear distance to gland center as a function of the standard deviation of the case-wise mean gland lumen size coded by consensus grade (major pattern) for each case.
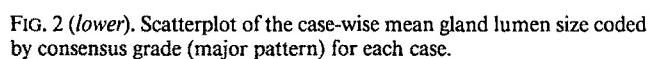
FIG. 2 (*lower*). Scatterplot of the case-wise mean gland lumen size coded by consensus grade (major pattern) for each case.

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TABLE 6. NONPARAMETRIC KRUSKAL-WALLIS TEST OF ARCHITECTURAL FEATURES FOR GLEASON GRADE 2 *VERSUS* GLEASON GRADE 3 GROUP AFTER CORRECTING FOR TIES

Feature	Chi Square	Significance
Mean nearest glandular distance	18.70	0.00
SD of number of glands per field	17.59	0.00
Mean nuclear distance to the center of gland	16.72	0.00
Mean number of glands per field	16.14	0.00
Mean gland size	15.08	0.00
Mean nuclear count per gland	9.72	0.00
Mean roundness of gland	9.00	0.00
Mean solid nuclei count per field	9.00	0.00
SD of roundness of gland	6.85	0.00
SD of solid nuclei count per field	5.82	0.01
SD of gland size	4.12	0.04

SD = standard deviation.

slide could be explained by the presence of an approximately equal mixture of patterns.

To measure glandular size, the Gleason grade 2 and 3 groups were separated at 2,200 μm^2 with the use of the cell image analysis system. Those considered to be in the "borderline" zone by objective measurements did not have a unanimous subjective interpretation, being assigned as Gleason grade 3 by some and grade 2 by others.

In conclusion, there appears to be a direct relationship between increasing Gleason grade and increasing gland variability, and an inverse relationship between Gleason grade and the gland luminal area and number of glandular nuclei, as assessed by a consensus group of pathologists. However, because the number of cases in this study was limited, the findings should be interpreted with caution. Additional studies, using high-resolution nuclear measurements, may be useful in identifying cases that appear
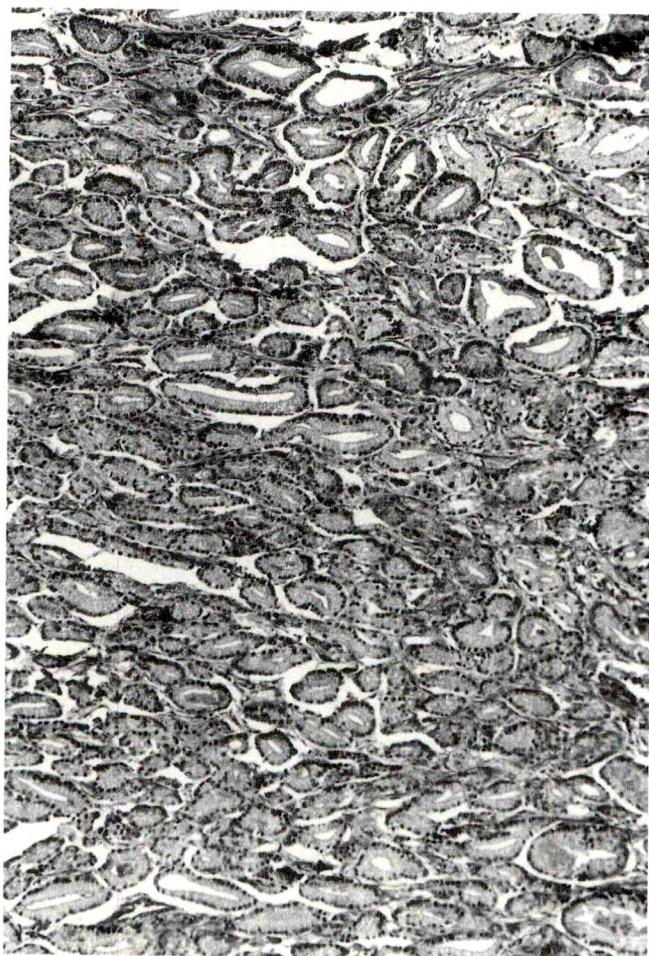
FIG. 3 (*left*). Adenocarcinoma of prostate with closely admixed Gleason grade 2 and 3. This tumor was interpreted by the individual pathologists to be either a Gleason grade 2 or 3. Hematoxylin and eosin (×40).
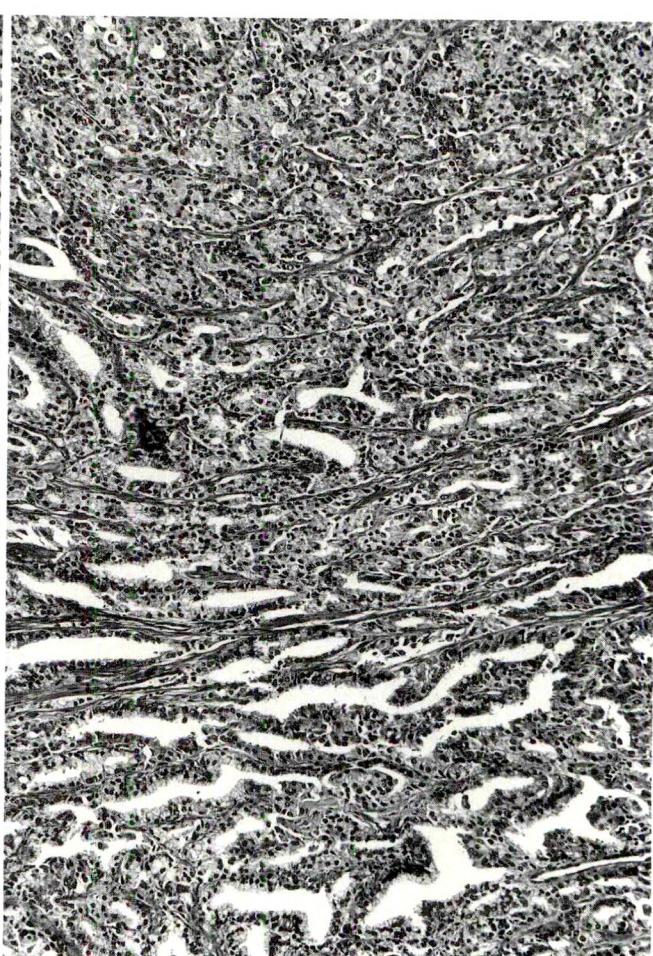
FIG. 4 (*right*). This Gleason grade 3 tumor has large glands on the right and very small glands on the left. This would lead to an overall interpretation of intermediate size glands based on an *average* of gland sizes in the tumor. Hematoxylin and eosin (×40).
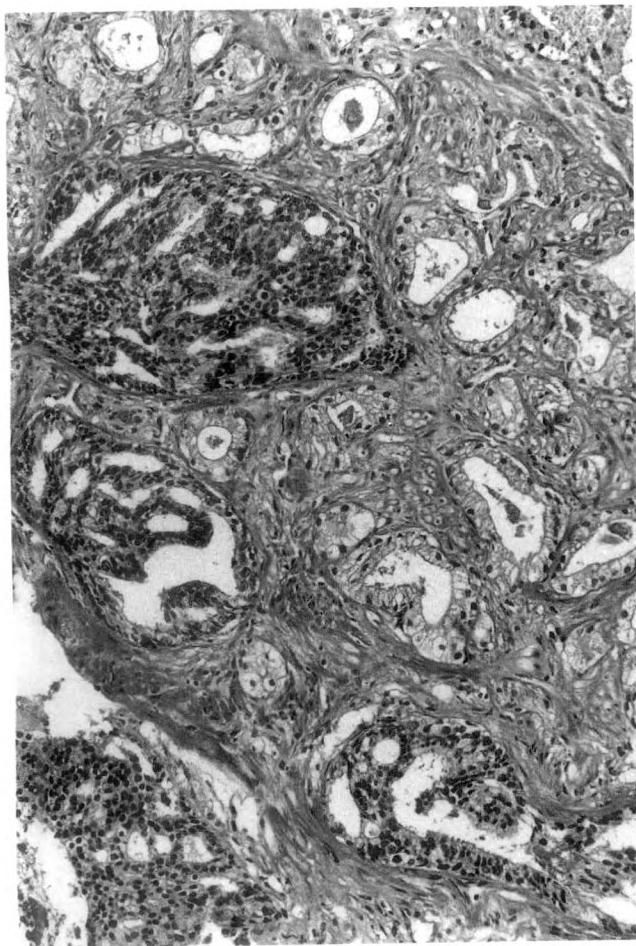
FIG. 5. This tumor has a cribriform pattern resulting in mostly small, variable lumens but large and regular outlines. Hematoxylin and eosin (×100).

to be "borderline" on objective measurements, as well as in subjective evaluation. We are developing an automated system that will simultaneously evaluate nuclear and architectural features, as well as subjective clues, which can be used in grading prostate tumors.[11]

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Original Article

Diagnostic Problems in Tissues Previously Sampled by Fine-needle Aspiration

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Unusual tissue changes in needle tracts after fine-needle aspiration were studied in two specimens. One specimen, a 3.0×2.5 cm mesenteric nodule, was found six days after a nondiagnostic transabdominal fine-needle aspiration of a retroperitoneal mass. At frozen-section examination, the nodule had proliferating spindle-shaped cells interspersed with adipocytes that raised the possibility of well-differentiated liposarcoma. On permanent sections, however, there was fat necrosis and reactive spindle-shaped cells that immunohistochemically were consistent with myofibroblasts. In the second case, a needle tract was noted in breast tissue on which a biopsy was performed two days after a cytologic diagnosis of carcinoma. The 0.4-mm-wide tract contained neutrophils, foamy histiocytes, and clumps of intact, atypical epithelial cells without a desmoplastic or elastotic component. Aside from these epithelial cells, nothing in the biopsy specimen suggested invasive carcinoma. The cells were identical to those from a comedocarcinoma present in the biopsy and mastectomy specimens. These two cases provide examples of pitfalls that may arise during the examination of biopsy specimens obtained after fine-needle aspiration. (Key words: Fine-needle aspiration; Frozen section; Tissue reaction; Needle tract) Am J Clin Pathol 1991;96:76–80

Fine-needle aspiration (FNA) is a well-established and safe method for the rapid diagnosis of palpable and deep-seated masses.[1] Few clinically important complications—including bleeding, pneumothorax,[2] infections and inflammatory reactions,[3] and death[4]—result from FNA. Some studies also have reported tumor seeding in needle tracts.[5–8] Little attention has been focused on tissue changes resulting from FNA. Three studies described FNA-induced changes in thyroid nodules,[9–11] and two others[12,13] examined the effects of FNA on lymph node histologic characteristics. We report two cases in which the tissue changes induced by FNA made the histologic sections difficult to diagnose.

REPORT OF TWO CASES

Case 1

A 38-year-old man had right groin pain radiating to the flank. A right-sided retroperitoneal mass impinging on the inferior vena cava was seen

on computed tomography (CT) scan. Four CT-guided, 22-gauge needles were placed in adjoining locations in the mass. The smears revealed a few atypical cells of uncertain type, but their paucity precluded diagnosis. Six days later, an exploratory laparotomy was performed. A 3.0×2.5 cm nodule in the small bowel mesentery and a 10.0×5.0 retroperitoneal mass extending from the head of the pancreas to the aortic bifurcation were observed. The mesenteric nodule was sent for frozen-section diagnosis, followed by biopsy specimens of the retroperitoneal mass. On frozen-section examination of the mesenteric nodule, the possibility of a well-differentiated liposarcoma was considered, but a firm diagnosis was deferred. After the procedure, the surgeon stated that the mesenteric nodule occurred in the area of the prior FNA; therefore, it became evident that this represented a reactive process. After debulking and subsequent histologic examination of the retroperitoneal mass, a diagnosis of metastatic seminoma was established. Ultrasonographic examination identified a 2-cm nodule of the right testicle. After orchiectomy, microscopic examination of the testicular tumor revealed a seminoma. The patient was treated with postoperative chemotherapy.

Case 2

A 49-year-old postmenopausal woman with a family history of breast carcinoma had a bloody discharge from the left nipple. An 8-cm left breast mass was found on physical examination. FNA of the breast mass was performed at another institution, and ductal carcinoma was diagnosed. The patient was referred to the University of Virginia Health Sciences Center. A biopsy of the left breast was performed two days after FNA, and the specimen contained extensive intraductal carcinoma, without areas of invasion. Because of the extent of the disease, a left modified radical mastectomy with axillary node dissection was performed.

PATHOLOGIC FINDINGS

Case 1

Grossly, the mesenteric nodule consisted of several fragments of firm tan tissue, with an aggregate dimension of 3.0 × 2.5 cm. Microscopic examination revealed loosely arranged, plump, spindle-shaped cells infiltrating the adipose tissue (Fig. 1). These cells had large nuclei with fine, even chromatin and prominent nucleoli (Fig. 2). Several mitotic figures were identified. There were foci of histiocytes with finely vacuolated cytoplasm (Fig. 3). These features raised the possibility of well-differentiated liposarcoma on frozen-section examination. In the permanent sections, lymphocytes and plasma cells were intermixed and there were obvious foci of fat necrosis. Immunohistochemical studies using the avidin–biotin–peroxidase complex technique on paraffin-embedded sections were performed with antibodies to vimentin (V9; prediluted; DAKO, Santa Barbara, CA), muscle-specific actin (HHF-35; 1:4,000; Enzo Biochemical, New York, NY), keratin (CAM 5.2; prediluted; Becton Dickinson, Mountain View, CA), desmin (prediluted; BioGenex, Dublin, CA), and S-100 protein (polyclonal; prediluted; BioGenex). The chromogen was 3,3′ diaminobenzidine tetrahydrochloride. The spindle-shaped cells were positive for vimentin and muscle-specific actin but negative for keratin, desmin, and S-100 protein. This immunohistochemical profile is consistent with myofibroblastic differentiation.

The retroperitoneal and testicular masses contained seminoma. This case provides an example of an unusual tissue reaction after FNA, in which the lesion mimicked a well-differentiated liposarcoma on frozen-section examination.

Case 2

The breast biopsy specimen consisted of three pieces of fibrofatty tissue with an aggregate dimension of 4.7 × 1.9 × 1.0 cm. Microscopically, there was extensive intraductal carcinoma. The cells were large and had variably sized nuclei with finely granular chromatin and prominent nucleoli. Comedonecrosis and calcifications were seen in several ducts. Two of the histologic sections showed a 0.4-mm-wide linear tract containing numerous histiocytes, a few neutrophils, lymphocytes, and scattered red blood cells (Fig. 4). Within this inflammatory background, there were clusters of intact abnormal epithelial cells (Figs. 5A and B) that were identical to those present in the adjacent foci of intraductal carcinoma (Fig. 6). A desmoplastic response and elastosis were absent. The question of invasive carcinoma was considered. It was believed, however, that the epithelial cells probably were dislodged from the intraductal carcinoma, simulating an infiltrating tumor.

The mastectomy specimen contained a 7.5 × 6.0 cm mass deep to the previous biopsy site. On cut surface, the mass was firm and gritty and had an infiltrative appearance with yellow streaks. Histologic examination revealed an extensive intraductal carcinoma involving approximately 75% of the ducts. There was also an infiltrating component that constituted approximately one-third of the mass. The cells of the infiltrating ductal carcinoma were embedded in a desmoplastic stroma, unlike the groups of atypical cells seen in the needle tract in the biopsy specimen. The axillary lymph nodes were free of carcinoma.

DISCUSSION

Although FNA is a common procedure, the resulting histologic changes rarely are identified or examined in excised tissues. Tissue reactions produced by needles are apparent when biopsy or resection is performed a few days after FNA.[11,12]

Difficulties in histologic interpretation that result from changes produced by FNA are unusual, except in lesions involving the thyroid gland.[9-11] In a series of 30 thyroid tissue specimens for which the biopsies were preceded by FNA, Jones and associates identified two cases in which changes were evident histologically that were believed to have resulted from FNA.[9] In one of their cases, almost total infarction of a thyroid nodule was attributed to FNA. The lesion was believed to represent a malignant neoplasm, but it could not be classified because there was only a scant amount of viable tumor. Kini and Miller described complete tumor infarction in 16 of 1,150 thyroid nodules that had been sampled by FNA.[10] Hemorrhagic nodule was the most common diagnosis when there was no known history of FNA. In 12 instances, the FNA specimen showed a Hürthle cell neoplasm, and, in the other 4 cases, papillary carcinoma had been diagnosed in the FNA smears.

In a recent study, LiVolsi and Merino examined 64 thyroid nodules that were removed after FNA.[11] In four of ten infarcted nodules, diagnostic interpretation was impeded by the extensive necrosis. Two follicular adenomas had epithelial cells in the lesion's capsule that simulated invasion, but it was found that the cells were in continuity with the needle tract. Hemorrhage and vascular alterations in another adenoma were believed to mimic angiosarcoma. These authors also described the acute histologic changes (one to three weeks after FNA) that resulted from FNA, including hemorrhage, tissue destruction, mitotic figures in endothelial and epithelial cells, granulation tissue formation, and capsular distortion. Chronic tissue changes (more than three weeks after FNA) included fibrosis, hemosiderin-laden macrophages, trap-
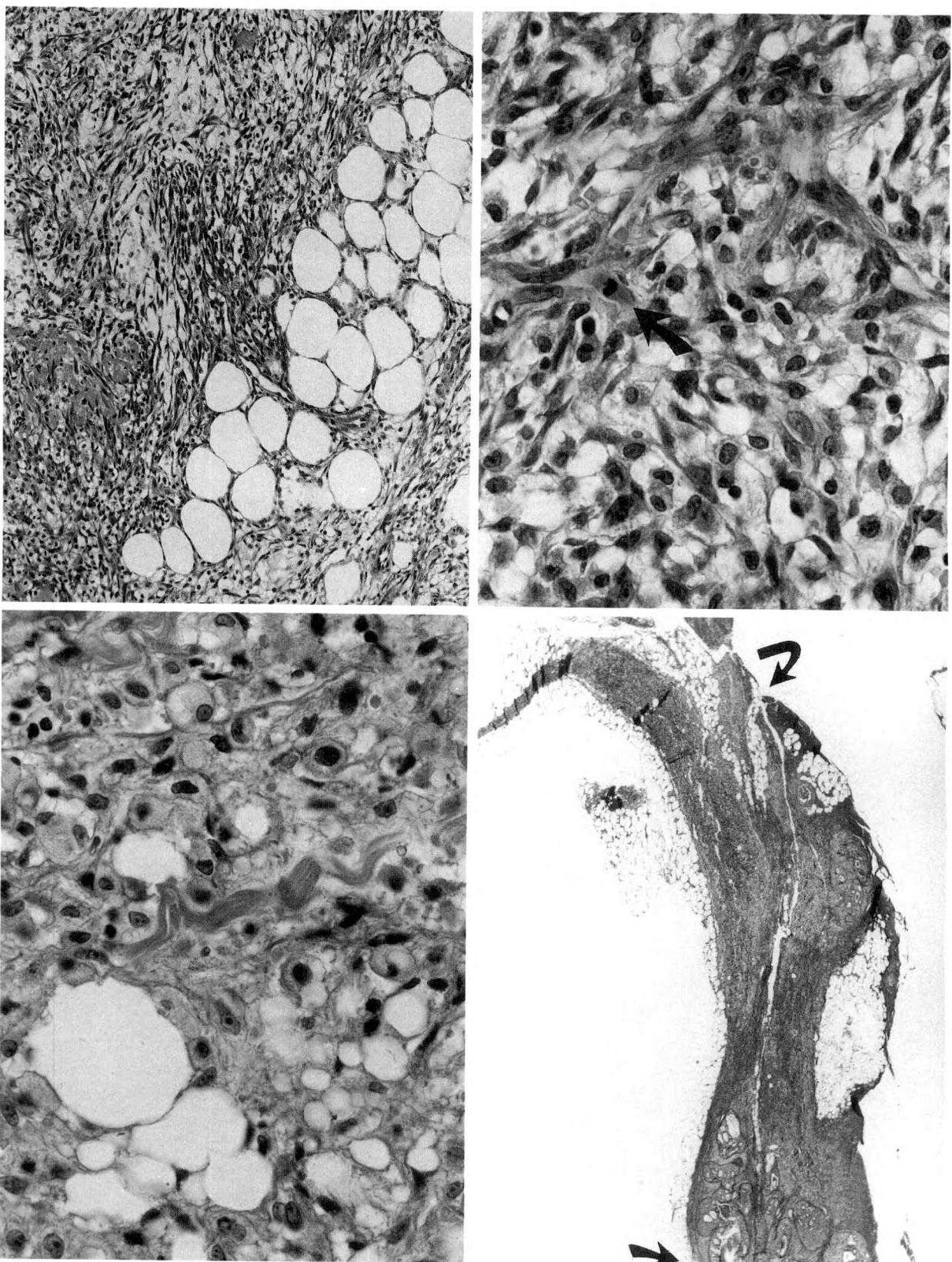
FIG. 1 (*upper left*). The mesenteric nodule contained mature adipose tissue and broad bands of spindle-shaped cells. Hematoxylin and eosin stain (×125).

FIG. 2 (*upper right*) Loosely arranged, plump spindle-shaped cells had enlarged nuclei, fine chromatin, and prominent nucleoli. Mitotic figures were present (arrow). Hematoxylin and eosin stain (×500).

FIG. 3 (*lower left*). Epithelioid cells in the areas of fat necrosis had finely vacuolated cytoplasm. Hematoxylin and eosin stain (×500).

FIG. 4 (*lower right*). At very low magnification, the needle tract appears as a cleft in the tissue that runs from the edge of the specimen (bent arrow) to a large focus of intraductal carcinoma (arrows). Hematoxylin and eosin stain (×12).
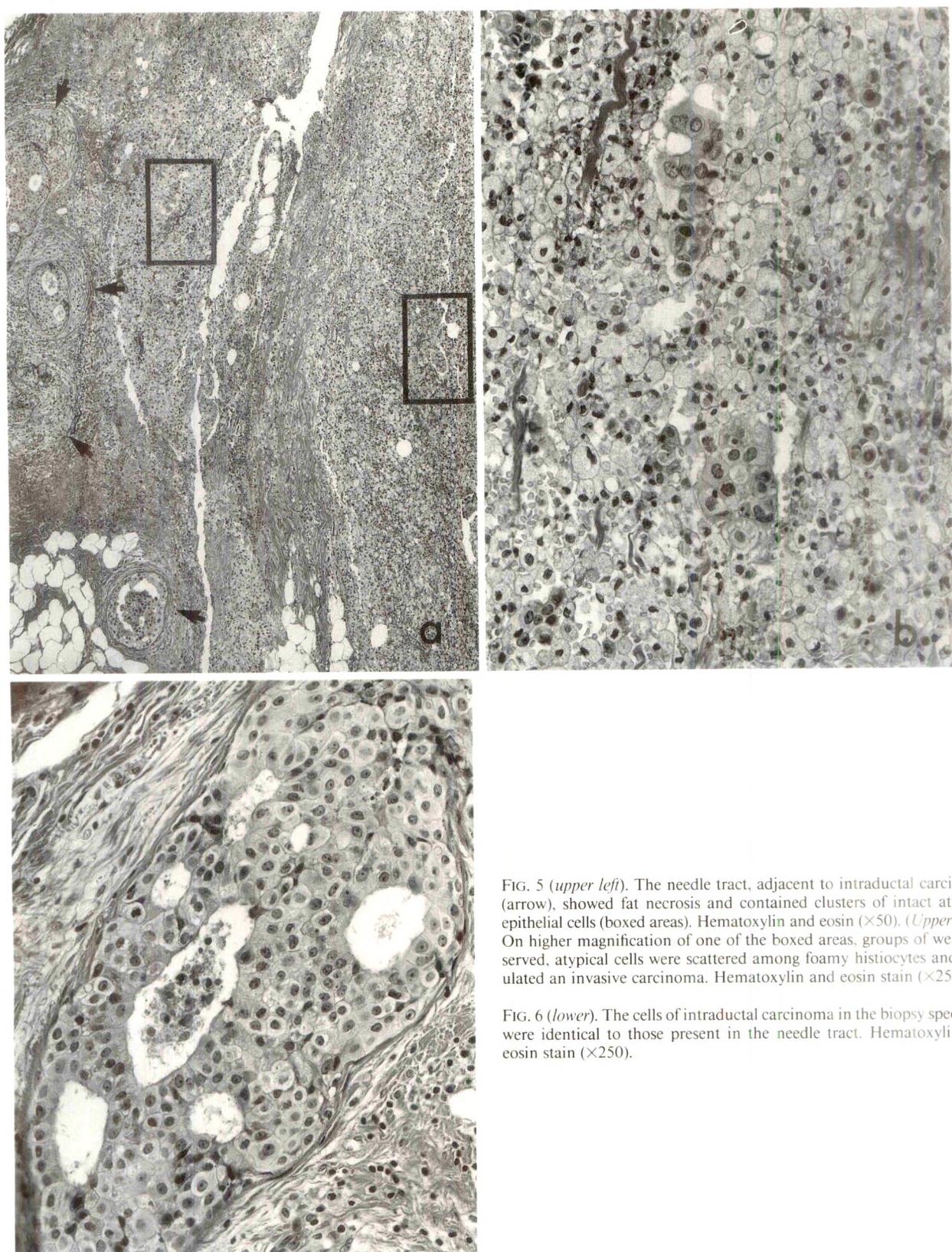
FIG. 5 (*upper left*). The needle tract, adjacent to intraductal carcinoma (arrow), showed fat necrosis and contained clusters of intact atypical epithelial cells (boxed areas). Hematoxylin and eosin (×50). (*Upper right*) On higher magnification of one of the boxed areas, groups of well-preserved, atypical cells were scattered among foamy histiocytes and simulated an invasive carcinoma. Hematoxylin and eosin stain (×250).

FIG. 6 (*lower*). The cells of intraductal carcinoma in the biopsy specimen were identical to those present in the needle tract. Hematoxylin and eosin stain (×250).

ping of epithelium in the nodule's capsule, nuclear atypia, and squamous and oncocytic metaplasia.

In describing the effects of FNA on lymph nodes, Behm and colleagues concluded that the changes produced by needle aspiration did not interfere with histologic evaluation.[12] They limited their material to benign lymph nodes, however, and were interested primarily in documenting the architectural preservation. FNA produced the following early changes: hemorrhage, foamy histiocytes, fibrin, neutrophils, and lymphocytes. The later changes included a healed, fibrotic linear tract.

In our cases, the changes produced by FNA caused diagnostic problems. In case 1, the problem involved an intraoperative consultation specimen. The frozen section of a biopsy specimen from a mesenteric mass demonstrated a proliferation of spindle-shaped cells intermixed with the mature adipose tissue, an appearance reminiscent of well-differentiated liposarcoma. Because of the retroperitoneal mass, a diagnosis of metastatic sarcoma was considered. The unusual histologic appearance of the nodule resulted from fat necrosis and myofibroblastic proliferation. In the second case, the fine-needle tract in a breast biopsy specimen contained well-preserved malignant cells that mimicked an infiltrating carcinoma; however, the linear orientation and the composition of the surrounding reaction were strongly suggestive of recent trauma produced by FNA. This change differed from the desmoplastic stromal reaction seen in invasive tumors and was quite dissimilar to the infiltrating cancer found in an adjacent part of the breast. These two cases emphasize that the awareness of a previous FNA may help preclude an erroneous histologic diagnosis.

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Original Article

Composite Lymphoma

A Clinicopathologic Analysis of Nine Patients with Hodgkin's Disease and B-Cell Non-Hodgkin's Lymphoma

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Nine patients had composite lymphoma in which Hodgkin's disease (HD) and non-Hodgkin's lymphoma (NHL) involved the same anatomic site. Two of these patients had relapses of their tumors. In one, the initial biopsy specimen contained follicular and diffuse large cell NHL with unclassifiable HD, but the relapse showed diffuse large cell NHL with nodular sclerosis HD. In the other patient, both biopsy specimens showed follicular mixed NHL; the HD component in the initial biopsy specimen was nodular sclerosis, whereas, at relapse, it had the appearance of interfollicular HD. In the remaining seven patients, the HD component was subclassified as nodular sclerosis (three specimens) or mixed cellularity (three specimens), or it was unclassifiable (one specimen). The NHL component was categorized as diffuse large cell (two specimens), diffuse large cell immunoblastic (two specimens), follicular and diffuse large cell (one specimen), diffuse mixed small and large cell (one specimen), and lymphocytic lymphoma of intermediate differentiation (modified Rappaport classification) (one specimen). Paraffin section immunoperoxidase studies were done on the NHL component in eight patients (nine specimens) and on the HD component in six patients (seven specimens). In each of these, the NHL component was leukocyte common antigen (LCA) positive and Leu-M1 negative. In addition, the neoplastic cells were L26 positive and UCHL-1 negative, indicating a B-cell phenotype. In five of seven immunophenotyped cases, Reed-Sternberg (RS) and Hodgkin's (H) cells from the HD areas were Leu-M1 positive and LCA negative, reflecting an immunophenotype that is typical of non–lymphocyte-predominant HD. In two specimens, the malignant cells were negative for Leu-M1 and LCA (with positive internal controls). Composite lymphomas composed of HD and NHL are unusual, and cases of coexistent HD of the non–lymphocyte-predominant subtype and NHL are even less common. The results of the current study and a review of the literature indicate that this phenomenon usually involves a B-cell NHL that coexists with HD, perhaps further suggesting a close relationship between the malignant cells of HD (RS and H cells) and B lymphocytes. (Key words: Malignant lymphoma; Composite lymphoma; Hodgkin's disease; Immunohistochemistry) Am J Clin Pathol 1991;96:81–89

"Composite lymphoma" has been defined as a tumor showing two distinct histologic subtypes of malignant lymphoma that involve the same lymph node or extranodal anatomic site.[1,2] Most often, composite lymphomas are composed of two separate types of non-Hodgkin's lymphoma (NHL).[1,3] Since the development of immunophenotypic[4,5] and genotypic techniques,[6,7] most composite lymphomas have been recognized as bimorphic expressions of the same neoplastic clone. For example, in lymph nodes involved by follicular lymphoma and diffuse large cell lymphoma, both components commonly express surface immunoglobulin of the same light chain class and presumably belong to the same clone.[5] Genotypic studies of one composite lymphoma, in which follicular lymphoma and lymphoblastic lymphoma coexisted, demonstrated that both neoplasms had identical immunoglobulin gene rearrangements, as well as the t(14;18) chromosomal translocation.[7] The lymphoblastic lymphoma in this case also had a c-myc rearrangement.[7] Thus, such cases are believed to represent two different histologic expressions of the same progenitor cell population, with evolution into an aggressive neoplasm.

Less frequently, composite lymphomas have been reported in which Hodgkin's disease (HD) and NHL coexist. Traditionally, this occurrence has been considered coincidental because HD and NHL are believed to represent mutually exclusive clinicopathologic entities.[1,8,9] However, cases of nodular lymphocyte-predominant HD and large

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cell lymphoma were documented recently in which areas of transition between the two neoplasms were apparent.[10] In addition, the malignant cells in both tumors expressed the leukocyte common antigen (LCA) (CD45) and pan-B-cell markers. Therefore, it was suggested that these two processes may be related.[10]

Occasionally, other subtypes of HD also have been found with NHL in the same anatomic site.[1,8,11–14] In this article, we report nine cases (two with recurrent neoplasms; 11 surgical specimens) of composite lymphoma containing HD (5 nodular sclerosis, 3 mixed cellularity, 1 interfollicular, and 2 unclassified) and NHL involving the same topographic location. In all patients, the NHL component had histologic features and/or an immunophenotype that were consistent with B lineage. This clinical phenomenon may support the lymphoid origin of the Reed-Sternberg (RS) and Hodgkin's (H) cells of HD. In addition, the occurrence of HD with B-cell NHL may suggest that, in at least some cases, RS and H cells are of B-lymphoid origin or that they may arise from a precursor cell that is shared with B-lymphocytes.

MATERIALS AND METHODS

All 11 specimens (from nine patients) were obtained from the files of the Laboratory of Pathology of the National Cancer Institute, for the years 1975 through 1989. In each case, HD and NHL occurred in the same anatomic site. Cases of composite chronic lymphocytic leukemia and HD were excluded from analysis and have been reported separately.[15]

Hematoxylin and eosin–stained histologic slides of formalin- or B5-fixed paraffin-embedded tissue were prepared at the submitting institutions. In 9 of 11 specimens, immunoperoxidase studies were performed with the use of fixed, paraffin-embedded sections and an avidin–biotin complex technique, as described previously.[16] The antibody panel included LCA (CD45), UCHL-1 (CD45 RO), L26 (CD20) (DAKO, Santa Barbara, CA), and Leu-M1 (CD15; Becton Dickinson, Mountain View, CA). The Leu-M1–positive, LCA-negative phenotype was considered to be consistent with that of RS and H cells of the nodular sclerosis, mixed cellularity, and lymphocyte-depleted subtypes of HD.[17] The pan-B-cell antibody L26 may stain RS and H cells, usually in small numbers, in 10–50% of cases of HD.[18] The LCA-positive, Leu-M1–negative phenotype was considered to be typical of NHL. L26 positivity and UCHL-1 negativity in cells with this phenotype were considered to indicate evidence of B-cell lineage.[18]

Immunophenotypic studies were performed on cell suspensions (case 8A) or frozen sections (case 9), using methods that have been described previously.[10,19] The an-

tibody panel included immunoglobulin heavy (IgG, IgA, IgM) and light (kappa, lambda) chains (Bethesda Research Laboratories, Bethesda, MD); the pan-B-cell markers B4 (CD19), B1 (CD20) (Coulter Clone,® Hialeah, FL), and Leu-14 (CD22; Becton Dickinson); and the T-cell antibodies T11 (CD2; Coulter), Leu-1 (CD5), Leu-2 (CD8), Leu-3 (CD4), and Leu-4 (CD3) (Becton Dickinson).

RESULTS

Clinical findings are summarized in Table 1. The median age of the patients was 54 years, with a range of 22–84 years. There were seven men and two women. Eight patients were white and one was Asian. Three patients (cases 2, 3, and 8) had follicular NHL before composite lymphoma appeared. There were no cases in which a prior history of HD was elicited. In six patients without a history of lymphoma, the disease was staged clinically. Three patients had low- (stage I and II) and three had high-stage (stage III and IV) disease; two patients (cases 1 and 5) had large cell NHL involving the bone marrow, and one (case 9) had generalized lymphadenopathy. Two patients had B (systemic) symptoms. Eight patients received treatment (six had chemotherapy with or without radiation therapy, one had radiation therapy alone, and one had surgical excision only).

The biopsy sites included cervical lymph nodes (four patients), inguinal lymph node (four patients), supraclavicular lymph node (one patient), submandibular lymph node (one patient), anterior mediastinum (one patient), and stomach (one patient). Two patients had recurrent composite lymphoma; in these cases, biopsy specimens of both the initial and relapse specimens were available.

Pathologic and immunophenotypic findings are summarized in Table 2. Representative cases are illustrated (Figs. 1–3). The NHLs were classified as diffuse large cell (cases 2B, 3, 7), diffuse large cell immunoblastic (cases 1 and 5), follicular and diffuse large cell (cases 2A and 4), diffuse mixed small and large cell (case 6), follicular mixed small cleaved and large cell (case 8A and B), and lymphocytic lymphoma of intermediate differentiation (modified Rappaport classification) (case 9). All NHLs had histologic or immunophenotypic features that were consistent with a B-cell phenotype, and all cases that were immunophenotyped were LCA positive, Leu-M1 negative, L-26 positive, and UCHL-1 negative. In two cases (cases 8 and 9), the NHL component expressed monotypic immunoglobulin in fresh tissue.

The HD component was classified as nodular sclerosis (cases 1, 2B, 4, 7, 8A), mixed cellularity (cases 3, 5, 9), or interfollicular (case 8B), or it was unclassifiable (cases 2A and 6). In two lesions (cases 5 and 7), immunophenotypic studies on the HD component were unsatisfactory prob-

Composite Lymphoma

TABLE 1. SUMMARY OF CLINICOPATHOLOGIC DATA

Case No.	Age/Race/Sex	History of Lymphoma	Clinical Stage	B Symptoms	Treatment	Follow-up
1	66/W/M	−	IV	−	Clyclophosphamide, vincristine, bleomycin	Lost
2A	43/W/M	+, DLC, 4 years previously, FML, 2 years previously	N/A	−	Multiagent chemotherapy, autologous bone marrow transplant	DOD 26 months after diagnosis of CL
2B		Relapse, 2 years later				
3	63/W/M	+, FMC 3 years previously	N/A	−	Surgery only	DOD 2 months after diagnosis of CL
4	62/W/F	−	I	−	Local radiation	CR, 27 months after diagnosis of CL
5	22/W/M	−	IV	+	Cyclophosphamide, cisplatinum; prednisone, BCNU	DOD 6 months after diagnosis of CL
6	84/A/F	−	I	−	Patient refused treatment	Lost
7	25/W/M	−	II	−	Multiagent chemotherapy	CR, 3 months after diagnosis of CL
8A	48/W/M	+, FMC 12 years previously	N/A	+	Radiation therapy; nitrogen mustard; vincristine; prednisone; procarbazine	Died of SNC lymphoma, 55 months after diagnosis of CL. No evidence of follicular lymphoma or Hodgkin's disease
8B		Relapse, 18 months later				
9	73/W/M	−	III	−	Chlorambucil	CR, 21 months after diagnosis of CL

W = white; A = Asian; M = male; F = female; DLC = diffuse, large-cell NHL; FMC = follicular, mixed-cell NHL; CL = composite lymphoma; SNL = small, noncleaved-cell lymphoma; DOD = died of disease; CR = clinical remission; N/A = not applicable.

TABLE 2. SUMMARY OF PATHOLOGIC AND IMMUNOPHENOTYPIC DATA

Case No.	Biopsy Site	NHL Diagnosis (%)*	NHL Cell Phenotype	HD Diagnosis (%)*	RS/H Cell Phenotype
1	Cervical lymph node	Large-cell immunoblastic (75%)	LCA+, L26+, LeuMl−, UCHL−1−	Nodular sclerosis (25%)	LeuMl+, LCA−, L26+, UCHL−1−
2A	Inguinal lymph node	Follicular and diffuse large-cell (NA)	LCA+, L26+, LeuMl−, UCHL−1−	Unclassified (NA)	LeuMl+, LCA−, L26+, UCHL−1−
2B	Supraclavicular lymph node	Diffuse large-cell (90%)	LCA+, L26+, LeuMl−, UCHL−1−	Nodular sclerosis (10%)	LeuMl+, LCA−, L26−, UCHL−1−
3	Stomach	Diffuse large-cell (90%)	LCA+, L26+, LeuMl−, UCHL−1−	Mixed cellularity (10%)	LeuMl−, LCA−, L26−, UCHL−1−
4	Inguinal lymph node	Follicular and diffuse large-cell (50%)	Not done	Nodular sclerosis (50%)	Not done
5	Cervical lymph node	Large-cell immunoblastic (75%)	LCA+, L26+, LeuMl−, UCHL−1−	Mixed cellularity (25%)	Not technically satisfactory
6	Cervical lymph node	Diffuse mixed small and large-cell (95%)	LCA+, L26+, LeuMl−, UCHL−1−	Unclassified (5%)	LeuMl+, LCA−, L26−, UCHL−1−
7	Mediastinum	Diffuse large-cell (50%)	LCA+, L26+, LeuMl−, UCHL−1−	Nodular sclerosis (50%)	Not technically satisfactory
8A	Cervical and inguinal lymph nodes	Follicular mixed small cleaved and large-cell (75%)	Monoclonal IgMk LCA+, L26+, LeuMl−, UCHL−1−	Nodular sclerosis (25%)	LeuMl+, LCA−, L26−, UCHL−1−
8B	Submandibular lymph node	Follicular mixed small cleaved and large-cell (90%)	Not done	Interfollicular (10%)	Not done
9	Inguinal lymph node	Lymphocytic lymphoma of intermediate differentiation (90%)	Monoclonal IgMλ LCA+, L26+, LeuMl−, UCHL−1−	Mixed cellularity (10%)	LeuMl−, LCA−, L26−, UCHL−1−

* Percentage of biopsy specimen involved by either NHL or HD.

NA = not applicable. In case 2A the NHL and HD components were intimately admixed.
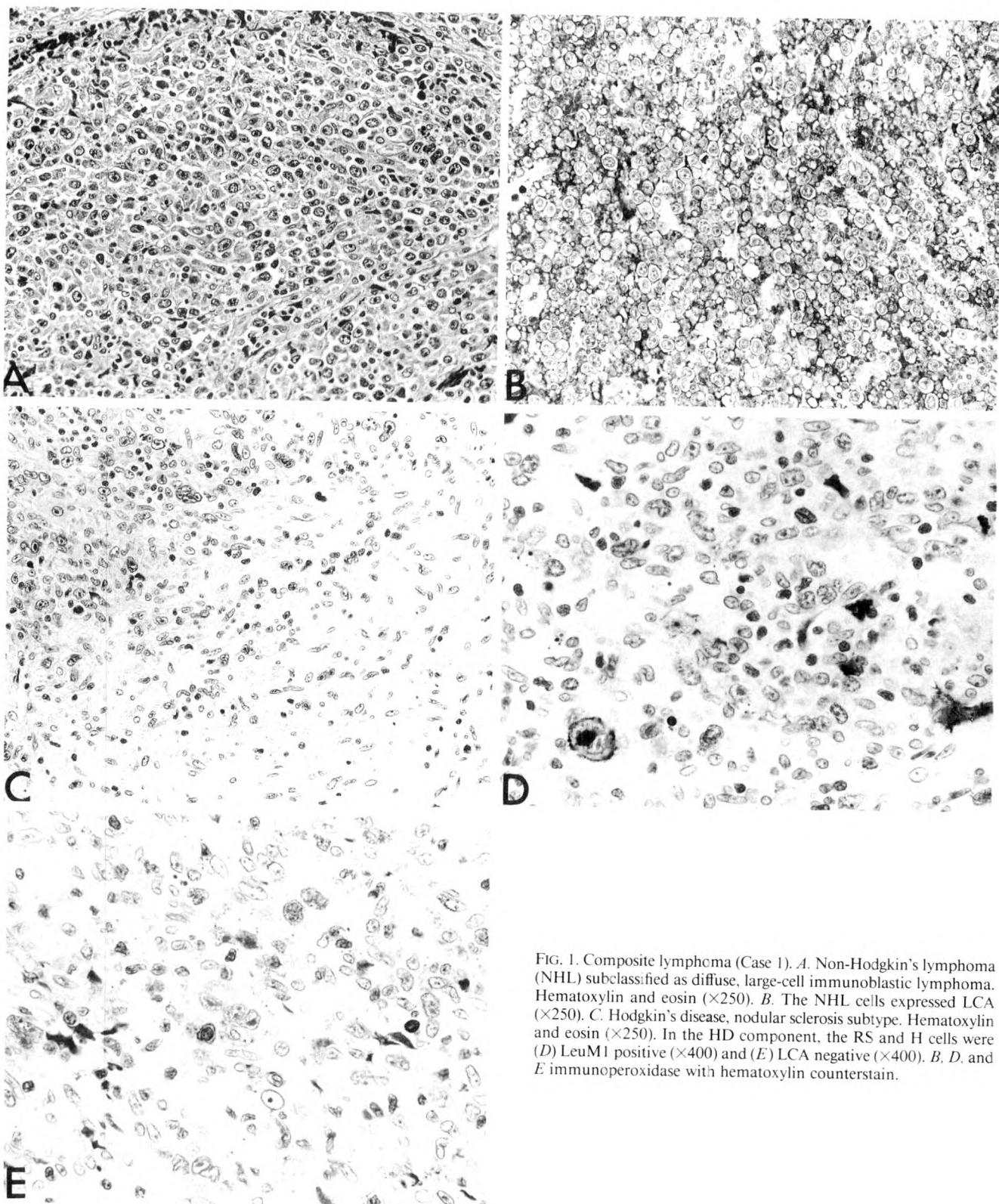
FIG. 1. Composite lymphoma (Case 1). *A.* Non-Hodgkin's lymphoma (NHL) subclassified as diffuse, large-cell immunoblastic lymphoma. Hematoxylin and eosin (×250). *B.* The NHL cells expressed LCA (×250). *C.* Hodgkin's disease, nodular sclerosis subtype. Hematoxylin and eosin (×250). In the HD component, the RS and H cells were (*D*) LeuM1 positive (×400) and (*E*) LCA negative (×400). *B, D,* and *E* immunoperoxidase with hematoxylin counterstain.
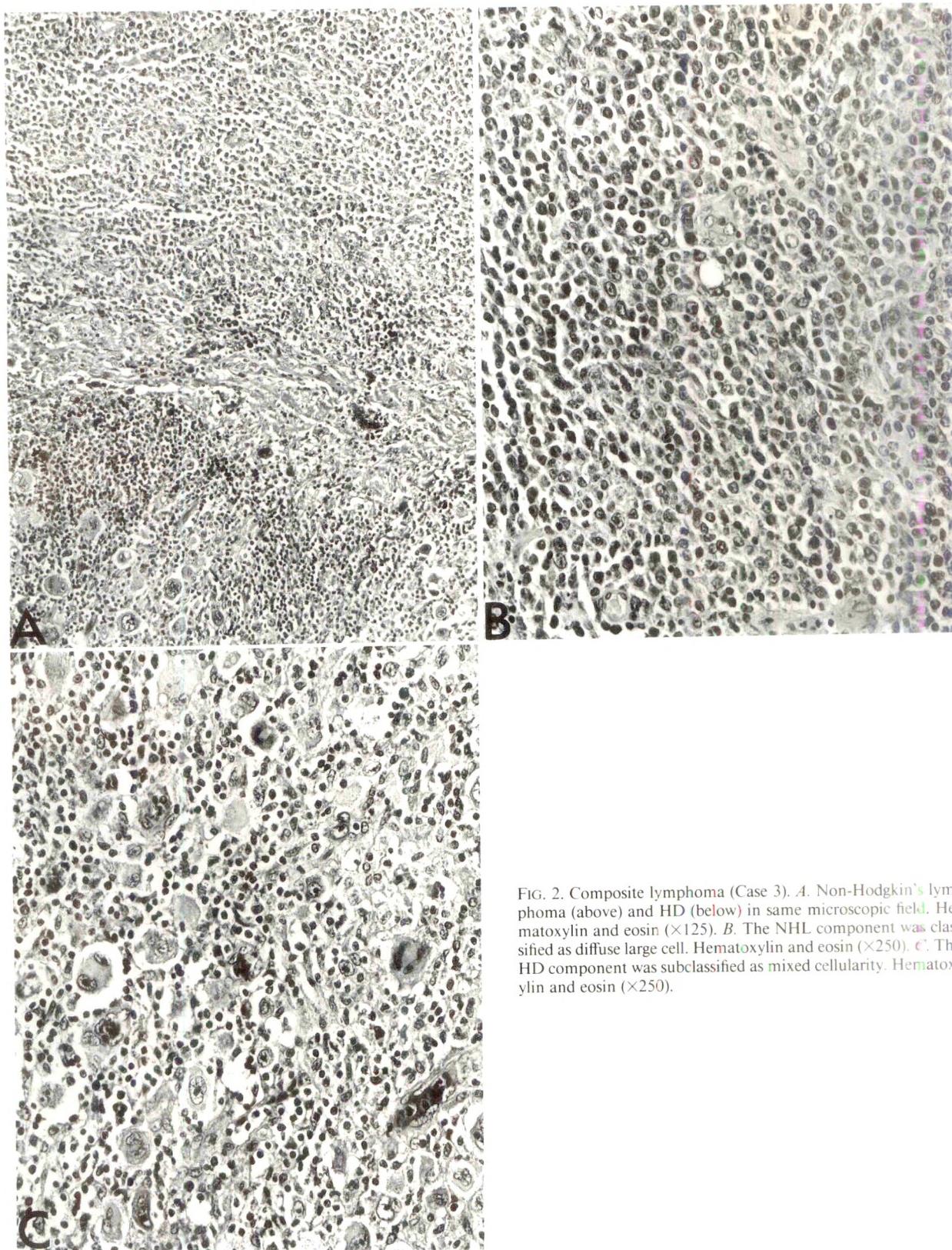
FIG. 2. Composite lymphoma (Case 3). *A*. Non-Hodgkin's lymphoma (above) and HD (below) in same microscopic field. Hematoxylin and eosin (×125). *B*. The NHL component was classified as diffuse large cell. Hematoxylin and eosin (×250). *C*. The HD component was subclassified as mixed cellularity. Hematoxylin and eosin (×250).
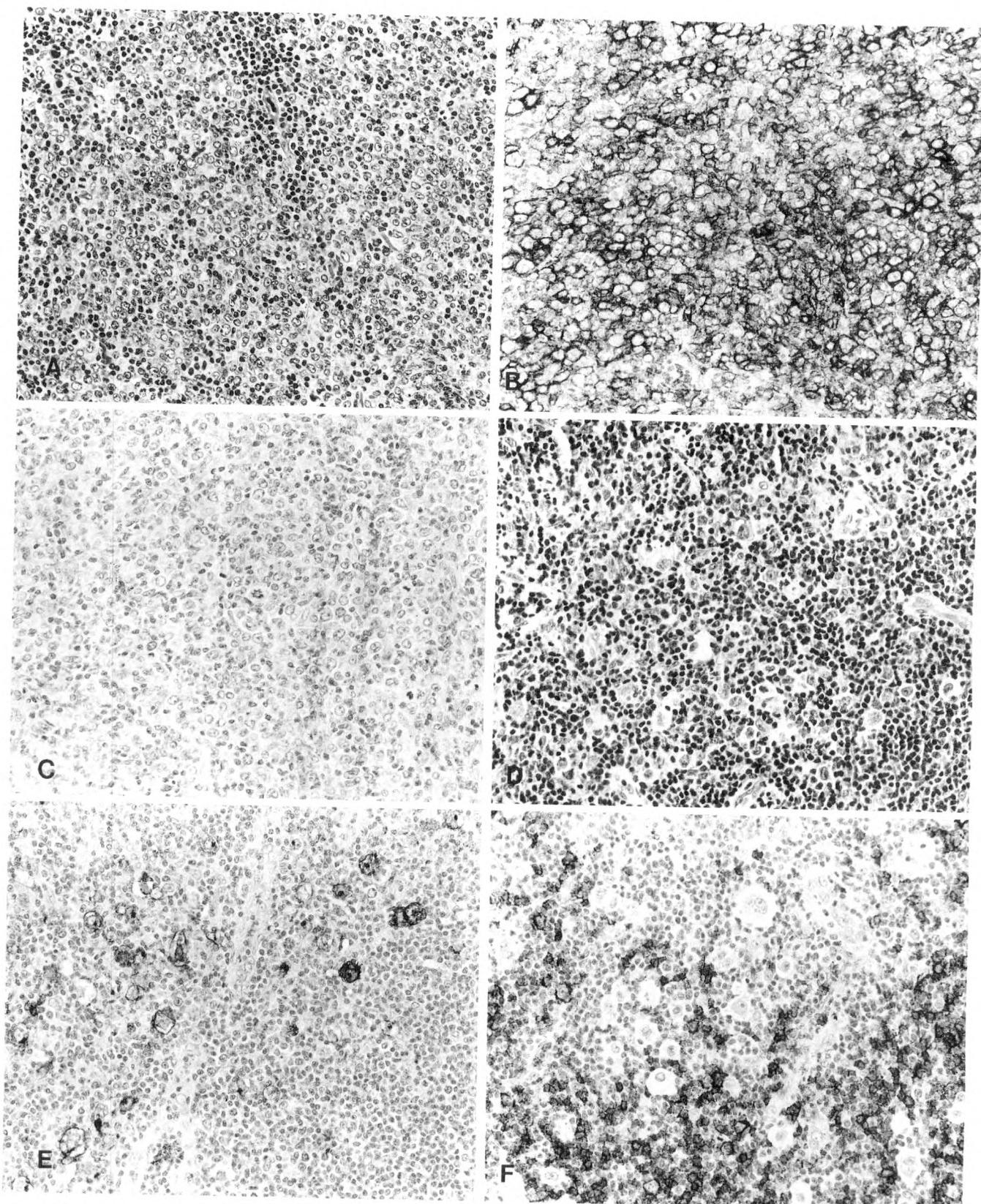
FIG. 3. Composite lymphoma (Case 4). *A.* Non-Hodgkin's lymphoma follicular large-cell type. Hematoxylin and eosin (×250). *B.* The NHL component was L26 positive, UCHL-1 negative (not shown), LCA positive (not shown), and (*C*) LeuM1 negative. *D.* Hodgkin's disease, nodular sclerosis type (hematoxylin and eosin, ×250) in which the RS and H cells were (*E*) LeuM1 positive, (*F*) L26 negative, and LCA negative (not shown). *B, C, E,* and *F.* Immunoperoxidase with hematoxylin counter stain (×250).

ably because of poor fixation (internal controls did not stain). In seven cases, the HD component was immunophenotyped adequately. In five of these, the RS and H cells were Leu-M1 positive and LCA negative, and, in two cases, they were Leu-M1 negative and LCA negative (with positive internal controls). In two cases (cases 1 and 2A), RS and H cells also expressed L26.

The NHL and HD components were distinct from each other in nine specimens, although there was a transitional zone between the components in some neoplasms. In two specimens, the NHL and the HD components were more admixed. Case 2A showed single RS cells (Leu-M1 positive, LCA negative), as well as foci of HD (RS and H cells with an inflammatory background) that were interspersed throughout a background of focally follicular, predominantly diffuse large cell NHL. In case 8B, the HD component was interfollicular, and it surrounded the follicles of a follicular mixed small cleaved and large cell NHL.

Clinical follow-up information was available for seven patients. Two patients (cases 1 and 6) were lost to follow-up. The median surveillance period was 19 months (range, 2–55 months). Three patients died of residual lymphoma (cases 2, 3, 5) and had a median survival of 15 months. A fourth patient (case 8) was treated successfully with radiation therapy and chemotherapy; 18 months later, he had a relapse, but his disease responded to additional therapy. Thirty-seven months later, however, the patient died of small noncleaved cell lymphoma. Three patients (cases 4, 7, and 9) are alive and in clinical remission (27, 3, and 21 months after diagnosis, respectively.)

DISCUSSION

We have described nine patients (two with recurrent disease; 11 surgical specimens) with composite lymphoma in which both HD (5 nodular sclerosis, 3 mixed cellularity, 1 interfollicular, 2 not subclassified) and B-cell NHL involved the same anatomic site. This is an unusual clinical occurrence, and most cases of this phenomenon, as reported previously in the literature, have not been evaluated with immunophenotypic studies. In this report, 9 of 11 specimens were studied with paraffin-section immunoperoxidase methods. The NHL and HD components had appropriate immunophenotypes in each neoplasm. Specifically, the NHL element was positive for LCA and negative for Leu-M1. In addition, the neoplastic cells were L26 positive and UCHL-1 negative, indicating a B-cell lineage. The HD component was studied adequately (internal controls were positive) in seven specimens. RS and H cells were Leu-M1 positive and LCA negative in five neoplasms, consistent with the phenotype of the atypical cells in HD of the non–lymphocyte-predominant sub-

types.[17] In two specimens, the atypical cells were negative for both Leu-M1 and LCA, whereas in two additional tumors the immunohistologic results were noncontributory (probably secondary to inadequate fixation).

The definition of composite lymphoma has evolved over time. As originally conceived by Custer and Bernhard,[12,20] this disease was defined as a combination of two different NHLs occurring in the same patient, either at different sites or involving the same location. Subsequently, Rappaport[2] and Kim and associates[1,21] modified the definition and included cases of coexistent HD and NHL. They required that both tumors occur in the same lymph node or extranodal site. By the latter definition, approximately 50 cases of composite lymphoma, composed of HD and NHL, have been reported to date,[1,4,8–14,21,22] with the most common combination being that of lymphocyte-predominant HD and diffuse large cell lymphoma.[1,8,10]

Traditionally, the occurrence of lymphocyte-predominant HD and NHL has been considered as the coincidental involvement by two separate diseases. However, Sundeen and associates[10] and others[23,24] have reported cases with areas of transition between both components, concluding that the NHL resulted from a histologic progression of lymphocyte-predominant HD. Their results extended the observations of other authors that suggested that patients with lymphocyte-predominant HD are at risk for subsequent development of large cell lymphoma. Therefore, more than a coincidental relationship may exist between the two neoplasms.[25,26] In addition, one of us (E.S.J.) has organized a registry of these cases, and approximately 35 cases have been reviewed that illustrate this clinical phenomenon. In all cases that were immunophenotyped, it has been demonstrated that the large cell component and the L and H cells of lymphocyte-predominant HD have a B-cell phenotype. These results provide additional support for the concept that nodular lymphocyte-predominant HD may be related closely to B lymphocytes, as suggested by others.[13,18,27–29]

Rarely, composite lymphomas have been reported in which HD of the non–lymphocyte-predominant subtype and NHL involve the same topographic site.[1,11,13,14] The current report describes 11 specimens from nine patients illustrating this phenomenon. It is not known whether this clinical phenomenon represents the chance occurrence of two separate neoplasms or, alternatively, two morphologic expressions of the same neoplastic clone. All the NHLs in this study showed immunophenotypic or histologic evidence of a B-cell phenotype. This group of cases may suggest that at least some cases of HD of the non–lymphocyte-predominant subtype are related closely to, or arise from, B lymphocytes.

Although this series did not include cases of chronic

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lymphocytic leukemia (CLL), one of US (E.S.J.) has reported previously on the coexistence of composite CLL and HD.[15] In addition, HD is being reported with increasing frequency in patients with CLL and has manifested itself as a Richter's-like transformation.[30] In cases that were studied immunophenotypically, the CLL has been of the B-cell type, whereas the RS and H cells have manifested a "Hodgkin's" phenotype (Leu-M1 positive, LCA negative).

A growing body of clinical, pathologic, and scientific evidence appears to support the hypothesis that the RS and H cells of HD derive from lymphoid cells and, in at least some cases, from B lymphocytes or precursor cells shared by B lymphocytes.[31] In our experience, we have not observed a composite lymphoma in which the components were HD and a T-cell lymphoma. Although HD has been described in patients with a T-cell malignancy, such as mycosis fungoides, it has not been observed in the same anatomic site. Similarly, our review of the literature revealed that composite lymphomas including an HD component occur almost exclusively with B-cell lymphomas. In cases that are not immunophenotyped, the B-cell nature of the NHL component often can be determined from the histologic subtype (*e.g.*, follicular lymphoma, small lymphocytic lymphoma, or CLL). Cases of well-established HD that terminate in a leukemic phase also have been documented, in which the circulating cells, which morphologically resembled RS cells, had a B-cell phenotype and showed immunoglobulin gene rearrangements.[32] Similarly, a cell line established from a patient with HD has expressed B-cell antigens and clonal karyotypic abnormalities.[33] Immunophenotypic analyses (of RS and H cells) using frozen-sections' have also demonstrated the expression of the pan-B-cell antigen CD20, as detected with the B1[34,35] and L26[18] antibodies in some cases.

More recently, cytogenetic and molecular studies have provided additional evidence suggesting that HD may be related closely to B lymphocytes. For example, cytogenetic analysis of tissues involved by HD has revealed clonal chromosomal abnormalities, including the t(14;18) (q32;q21) translocation, which typically is found in follicular lymphomas.[36] In our laboratory, Stetler-Stevenson and colleagues[37] found this translocation in as many as a third of cases of HD of all subtypes, using the polymerase chain reaction and amplification for the major breakpoint region of the bcl-2 gene on chromosome 18. Indeed, five of nine patients in this series had an NHL with a follicular component at some point in their clinical course. Finally, immunoglobulin gene rearrangements also have been identified in many cases of HD with numerous RS and H cells, using Southern blot analysis.[38-40] Therefore, these findings further suggest a close relationship between the RS cell and the B lymphocyte.

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Original Article

Aberrant MT2 Positivity Distinguishes Follicular Lymphoma from Reactive Follicular Hyperplasia in B5- and Formalin-Fixed Paraffin Sections

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Often, it is difficult to distinguish follicular lymphoma from reactive follicular hyperplasia histologically. Immunotypic evidence of monoclonality cannot be demonstrated routinely or reliably in routine paraffin-embedded sections. To determine whether a panel of monoclonal antibodies reactive with lymphoid cells in paraffin-embedded sections might be useful in distinguishing these confusing proliferations, the authors examined 45 follicular lymphomas and 30 follicular hyperplasias with the following antibodies: L26, B2, MT1, MT2, and UCHL-1. All sections were routine paraffin preparations from formalin- or B5-fixed tissue and were immunostained with the avidin–biotin immunoperoxidase technique. Ninety-two percent of the B5-fixed and 77% of the formalin-fixed lymphomas were MT2 positive. None of the reactive hyperplasias stained positively, and none of the other antibodies demonstrated consistent differences between these benign and malignant proliferations. MT2 marks interfollicular T cells and mantle-zone B cells in normal lymph nodes but does not mark normal germinal centers; this staining pattern is retained in reactive hyperplasia. However, paradoxically, in most follicular lymphomas the neoplastic germinal centers show aberrant MT2 positivity. The authors conclude that MT2 may be useful in distinguishing follicular lymphoma from follicular hyperplasia in paraffin sections. (Key words: Immunoperoxidase; MT2; Follicular lymphoma; Follicular hyperplasia; Paraffin sections; B5;) Am J Clin Pathol 1991;96:90–94

Occasionally, all surgical pathologists face the challenge of distinguishing florid follicular hyperplasia (FH) from follicular (nodular) lymphoma (FL).[1-7] In the mid-1920s, Brill and associates[8] and Symmers[9] described the morphologic features of FL but did not recognize that it was a neoplasm; they believed that it was some form of extreme lymphoid hyperplasia. Gall and associates recognized the neoplastic nature of the condition and proposed criteria for distinguishing FL from FH.[10] Later, Rappaport and colleagues refined the criteria of Gall and associates and identified some additional helpful features.[6] Nevertheless, no single morphologic feature differentiates these follicular proliferations; accurate diagnosis depends on the coordinated evaluation of many microarchitectural and cytologic features. Although not foolproof, Rappaport's criteria have served pathologists well over the last 30 years. Still, some follicular proliferations defy accurate morphologic analysis,[11] and, unless there is pathologic evidence of systemic disease, such as bone marrow infiltration, only time and the eventual outcome verify the true diagnosis. Because morphologic criteria lack some precision, investigators have used newer diagnostic modalities.

Follicular lymphoma can be diagnosed accurately by establishing that the proliferative nodules are monoclonal. Monotypic surface light chain staining within the nodules establishes their monoclonality.[12-15] In contrast, hyperplastic follicles demonstrate polytypic staining. Alternatively, monoclonality can be confirmed by the demonstration of immunoglobulin gene rearrangement.[16-19] FLs also have a characteristic karyotype[20] and express a unique oncogenic protein (bcl-2) associated with a specific chromosomal abnormality.[21] However, for consistent and reliable results, these techniques require fresh unfixed tis-

sue.[13,15] Unfortunately, biopsy specimens usually arrive in the pathology laboratory in fixative, usually formalin. Moreover, the newer molecular biology techniques are not routinely available in most pathology laboratories.

Although surface light chains are very difficult to demonstrate in formalin-fixed, paraffin-embedded sections, many B- and T-cell antigens are preserved in fixed tissue and detected readily with numerous new commercially available antibodies; these are reliable for immunotyping malignant lymphomas.[22–29] In this study, we evaluated a panel of antibodies that are reactive in paraffin sections to determine whether their immunostaining patterns could distinguish FL from FH.

MATERIALS AND METHODS

We retrospectively studied 45 formalin-fixed lymph nodes (from 45 patients) involved by FL and 30 formalin-fixed lymph nodes (from 30 patients) showing reactive FH. They were diagnosed by conventional light microscopic examination using the criteria of Rappaport and associates[6] and Nathwani and associates[4]; the selected cases were typical and were not diagnostic problems. Thirteen of the lymphomas also were fixed in B5. These were placed in B5 for three hours, transferred to unbuffered 10% formalin, and left in formalin overnight. All specimens were processed routinely in paraffin.

The tissues were immunostained using the avidin–biotin immunoperoxidase technique.[30] Primary antibody was omitted from negative controls. Details of the monoclonal antibodies are listed in Table 1. The diagnoses were coded, and follicles were evaluated by two observers and scored as positive or negative for each antibody. Equivocal cases were scored as negative.

RESULTS

In most cases, the immunostaining pattern observed with MT2 clearly distinguished FL from FH. Hyperplastic follicle centers were negative and were surrounded by strongly stained mantle zone cells (Fig. 1). Within the benign follicles, a light sprinkling of positive, normal, small round mature lymphocytes was seen; these marked as T cells with UCHL-1 and MT1. Occasional reactive follicle cells showed weak, patchy, cytoplasmic positivity.

In contrast, the FL nodules were MT2 positive in 35 of the 45 formalin-fixed nodes and in 12 of the 13 B5-fixed nodes (Fig. 2). Formalin-fixed sections showed staining heterogeneity within individual sections, and the extent and intensity of positivity varied from case to case. B5-fixed tissue stained more intensely, crisply, and uniformly than formalin-fixed sections. Tumor cells had a surface-membrane staining pattern; however, large tumor cells with recognizable cytoplasm (centroblasts) also demonstrated cytoplasmic positivity. In some cases, MT2 also stained variable numbers of interfollicular lymphoma cells. Usually these could be distinguished from normal interfollicular T cells by their irregular cleaved nuclei, which were larger than small mature lymphocytes.

The immunostaining patterns seen with all other antibodies were similar in FL and FH, with no useful differentiating features evident. In general, they reacted as expected: L26 and MB2 stained neoplastic and reactive follicles, whereas MT1 and UCHL-1 stained neither. Technically, B5-fixed tissue stained far better.

DISCUSSION

MT2 is a mouse monoclonal antibody that reacts with a 200- and 190-kD antigen present on normal nonactivated T lymphocytes and B-mantle zone lymphocytes.[29] It does not react with normal follicular center cells; however, it marks many diffuse B-cell lymphomas.[29,31] In their evaluation of various antibodies in paraffin sections, Poppema and associates[29] briefly mentioned that the monoclonal antibody MT2 reacted with FL cells but not with normal follicular cells in germinal centers. Our results confirm their observations and indicate that the monoclonal antibody MT2 may be helpful in distinguishing FL from FH.

Many of the criteria of Rappaport and colleagues for the diagnosis of FL also are found in FH. For example, in FL the follicles are classically uniform in size and shape,

TABLE 1. MONOCLONAL ANTIBODIES REACTIVE WITH T AND B CELLS IN PARAFFIN SECTIONS

Antibody	Dilution	Source	Molecular weight	Main Reactivity	References
L26	1/50	Dakopatts, Denmark	200 kd	Pan B-cell marker	22–24, 26
MB2	1/5	Biotest, Germany	200 kd	Pan B-cell marker	24, 25, 28, 29
MT1	1/5	Biotest,	190 kd	Pan T-cell marker	24, 28, 29
UCHLI	1/50	Dakopatts	195 kd	Pan T-cell marker	24, 28
MT2	neat	Biotest	220 kd	Pan T-cell marker Normal mantle zone B cells	29
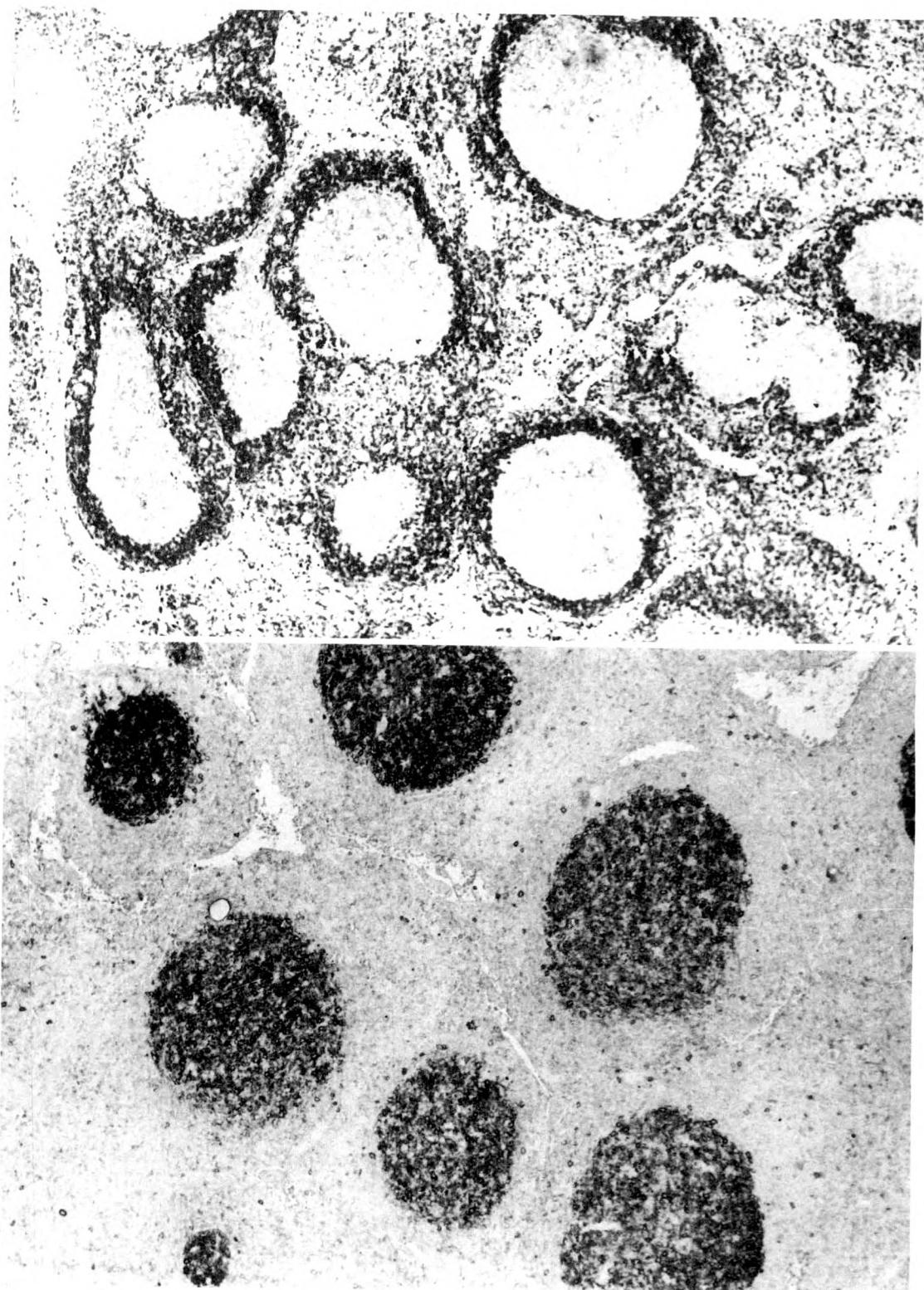
FIG. 1 (*upper*). MT2 staining of follicular hyperplasia showing negative germinal centers surrounded by strongly reactive mantle-zone cells. Occasional small normal T cells within the follicles also are positive. The T cells also were positive with MT1 and UCHL1. Original magnification (×40).

FIG. 2 (*lower*). MT2 staining of follicular lymphoma. Neoplastic follicle cells are positive. Original magnification (×40).

unlike reactive hyperplasia, in which the nodules vary in size and shape and often have an irregular serpiginous configuration. However, such confusing irregular nodules also are seen occasionally in lymphomas.[5] Nathwani and associates applied the criteria of Rappaport and associates to 80 patients and, using clinical follow-up to establish a final diagnosis, found that some of Rappaport's time-honored criteria, such as the size and shape of the follicles and the presence of extracapsular follicles, were not statistically significant in separating benign from malignant follicles.[4] In the study by Nathwani and colleagues, back-to-back, even distribution of follicles (regardless of size or shape) throughout the lymph node was the most important feature. However, this pattern occurred in only 85% of cases. The second most important feature was the number of follicles per unit area (low-power field area of 16.466 mm^2). Sixty or more follicles per unit area were found only in FL; this feature was present in 25% of FLs. The presence of interfollicular lymphoma cells also was valuable; these are often difficult to identify. Frequently, we found that these interfollicular tumor cells were easier to identify in MT2-stained sections than in hematoxylin and eosin–stained sections.

Although we believe that MT2 is valuable in distinguishing FL from FH, we recognize that our study has some limitations. We evaluated these immunostaining reactions in straightforward cases of benign and malignant follicular proliferations, cases that could be diagnosed readily by conventional light microscopic examination. We do not know whether the results would be so dramatic or as easy to interpret with genuine diagnostic problems. Our results should be regarded as preliminary, therefore, and should be confirmed by other laboratories with larger numbers of lymphomas. A recent study by Norton and associates[32] also confirmed the value of MT2; they found that 50% of formalin-fixed FLs were MT2 positive. Also, Ng and colleagues demonstrated MT2 positivity in 8 of 15 formalin-fixed FLs.[31]

We recommend the use of B5 fixation when possible; immunostaining with MT2 is crisp, easy to interpret, and far superior to staining after formalin fixation. The variable staining intensity and nonreactivity seen in some formalin-fixed sections could be caused by a number of technical factors, such as duration of fixation and whether or not the formalin was buffered (we find that unbuffered formalin is better for most immunostaining procedures). Some cases were processed in other laboratories, and we do not know all of the details regarding processing. We have observed that prolonged fixation in formalin (longer than 24 hours) reduces or abolishes immunoreactivity for MT2.

There are potential problems in the interpretation of MT2-positive nodules. FL should not be confused with small nodules of MT2-positive mantle cells in tangential sections through primary or reactive follicles; conventional histologic morphologic evaluation differentiates these; also, FLs are LN1 positive in B5-fixed tissue (and sometimes in formalin-fixed tissue), whereas mantle-zone nodules are LN1 negative.[33] Progressively transformed germinal centers and reactive lymph nodes showing follicular lysis contain MT2-positive cells and are a potential source of confusion; these should be differentiated, again, by careful morphologic evaluation. Usually these MT2-positive lymphocytes are small and strongly reactive, like normal T cells or mantle zone cells, and can be distinguished from adjacent groups of follicular center cells. We also have observed that the nodules in nodular lymphocyte–predominant Hodgkin's disease are MT2 positive. We have no experience with pseudonodular T-cell lymphomas[34,35] and do not know how these would react with MT2. Heterogeneity of staining poses a potential diagnostic problem. This is especially evident with tissue processed after prolonged formalin fixation; unfortunately, this limits the potential usefulness of MT2 immunoreactivity. With B5 fixation, staining heterogeneity is not a problem.

With the above caveats in mind, we recommend the following: If the follicles are nonreactive and the technical preparation is good, as evidenced by strong staining of residual mantle cells, the lesion probably is benign. If the follicles show equivocal staining or the technical preparation is inadequate, results cannot be interpreted reliably. If the follicles are clearly reactive with MT2, the diagnosis is FL.

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Original Article

The Predictive Value of Bone Marrow Morphologic Characteristics and Immunostaining in Primary (AL) Amyloidosis

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The authors previously demonstrated that bone marrow plasmacytosis in primary (AL) amyloidosis may be monoclonal or polyclonal. However, the clinical implications of the degree of plasmacytosis and its clonality have not been studied. The authors evaluated 62 patients with AL amyloidosis, 40 of whom had monoclonal medullary plasma cells. There was complete concordance between the light chain class of the plasma cells in the monoclonal cases and that of the circulating paraprotein in the 22 cases associated with a paraprotein. The remaining 22 patients had polyclonal plasma cells, although a paraprotein was detected in 6. The degree of plasmacytosis was significantly higher among patients with monoclonal plasma cells and correlated inversely with length of survival. The authors' findings indicate that the quantitation of bone marrow plasma cells in AL amyloidosis by immunoperoxidase studies may predict the clinical course. (Key words: AL amyloidosis; Clonality; Plasma cells; Immunoperoxidase) Am J Clin Pathol 1991;96:95–99

Primary amyloid of immunoglobulin light chain origin (AL) amyloidosis is associated with the deposition of amyloid fibrils, which have been shown to be structurally homologous to portions of immunoglobulin light chains.[1–12] This disorder often is associated with the presence of paraprotein in the serum or urine[3,8,9,12–16] and with an increased number of plasma cells in the bone marrow, even in the absence of clinically overt multiple myeloma.[17–20]

Previously, we studied bone marrow biopsy specimens from 45 patients with systemic amyloidosis to assess the degree of the medullary plasmacytosis and its clonality

(monoclonal vs. polyclonal).[21] Twenty-four of 35 patients with AL amyloidosis had monoclonal plasma cells, whereas 11 had polyclonal plasma cells. Five patients with secondary amyloidosis and five with familial amyloidosis had a mild polyclonal plasmacytosis. This study suggested that two subgroups of patients may have primary amyloidosis: patients with a monoclonal plasmacytosis representing part of the spectrum of plasma cell dyscrasias, and patients in whom a monoclonal plasmacytosis cannot be documented. However, the clinical implications of the degree of bone marrow plasmacytosis and the clonality of the medullary plasma cells have not been studied. We studied a larger series of patients with AL amyloidosis to assess the relationship of bone marrow morphologic and immunohistologic characteristics to the disease's behavior.

MATERIALS AND METHODS

Patient Population

Sixty-six cases of AL amyloidosis, for which bone marrow biopsy specimens or particle sections were available. were retrieved from the files of the Amyloid Research Center at Boston University Medical Center. The bone

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marrows were obtained between 1976 and 1989. All the patients had signs and symptoms related to organ involvement by amyloidosis. Their disease was classified as primary systemic amyloidosis on clinical and laboratory grounds. Patients with clinically overt multiple myeloma, as manifested by osteolytic bone lesions or generalized osteoporosis, hypercalcemia, or severe anemia, were excluded.[20,22]

Bone Marrow Studies

The bone marrow specimens were fixed in Zenker's fixative and processed routinely. Paraffin sections were stained with hematoxylin and eosin (H and E), periodic acid-Schiff reagent (PAS), and Congo red stain for amyloid. In 53 cases, Wright-Giemsa–stained bone marrow smears also were available. Sections for immunohistochemical studies were prepared from paraffin blocks. Cytoplasmic immunoglobulin light chains were demonstrated by the immunoperoxidase indirect sandwich technique, using a method published previously,[23] which was directed against kappa and lambda light chains (DAKOPATTS, Copenhagen, Denmark). Cases of known multiple myeloma with concordant paraproteins were used as positive controls.

Cell counts, performed on immunoperoxidase sections, and on bone marrow smears when available, were based on 200 nucleated cells or, in cases in which 200 cells were not available, on the total number of nucleated cells in the marrow space.

Statistical Analysis

Clinical and laboratory data were obtained from the files of the Amyloid Research Center and the patients' hospital records. For statistical analysis, the duration of the disease was defined as the length of time from the date of diagnosis to the date of the bone marrow biopsy. Survival time was calculated in two ways: from the date of diagnosis to the date of death, or to the present time, if the patient was still alive; and from the date of bone marrow biopsy to death, or to the present. Because of the skewed nature of the data for disease duration and survival, we used the median along with the mean ± standard deviation in presenting the data for these parameters.

All statistical analyses were done with SAS software (SAS Institute, Cary, NC). Data were compared by the analysis of variance (ANOVA) for the continuous data or chi-square analysis for the discrete (categoric) data. Scheffe's *post hoc* multiple testing was used, when necessary, for individual group comparisons once significance was established with ANOVA.[24] The survival analysis was conducted using Cox's proportional hazards regression model.[25] In all instances, a *P* value less than or equal to 0.05 indicated statistical significance.

RESULTS

Patient Population

Four cases were reclassified as multiple myeloma on the basis of sheets of anaplastic plasma cells in the bone marrow biopsy specimens. These cases were excluded from the study, leaving a total of 62 patients for evaluation. The patients included 34 men and 28 women, with an age range of 36.7–81.2 years (mean, 60.5 ± 10.4 years) at the time of bone marrow biopsy (Table 1). The duration of disease ranged from 0.0 to 5.8 years (median, 0.4; mean, 1.0 ± 1.3 years) at the time of bone marrow biopsy. Fifty-nine of the 62 patients were treated with colchicine; 8 of these patients also received melphalan and prednisone. Two of the remaining three patients were treated with melphalan and prednisone alone, whereas in one patient

TABLE 1. MORPHOLOGIC AND CLINICAL FEATURES OF 62 CASES OF AL AMYLOIDOSIS

	All 62 Cases (mean)	40 Monoclonal Plasma Cells (mean)	22 Polyclonal Plasma Cells (mean)
Sex	34 M 28 F	17 M 23 F	17 M 5 F
Age* (years)	36.7–81.2	37.2 –77.9	36.7–81.2
	(60.5 ± 10.4)	(59.3 ± 9.7)	(62.5 ± 11.6)
% Plasma cells	3–30%	3–30%	3–30%
	(10.5 ± 7.0%)	(12.1 ± 6.8%)	(7.5 ± 6.4%)
Paraprotein	28 cases	22 cases	6 cases
Duration† (years)	0.0–5.8	0.1–4.5	0.0–5.8
	(1.0 ± 1.3; median 0.4)	(0.8 ± 1.0; median 0.4)	(1.2 ± 1.7; median 0.6)
Survival‡ (years)	0.0–11.1	0.0–11.1	0.2–5.4
	(1.7 ± 1.8; median 1.2)	(1.6 ± 1.9; median 1.1)	(2.0 ± 1.5; median 1.7)
Survival§ (years)	0.1–11.3	0.1–11.3	0.1–9.0
	(2.7 ± 2.2; median 2.0)	(2.4 ± 2.1; median 1.9)	(3.2 ± 2.3; median 2.8)

* Age at time of bone marrow biopsy.
† Duration of disease from date of diagnosis to date of death or present.
‡ Length of survival from date of bone marrow biopsy to date of death or present.
§ Length of survival from date of diagnosis to date of death or present.

only the symptoms were treated. In January 1990, 18 of the 62 patients were alive. Survival length from the time of the bone marrow biopsy ranged from 0.0 to 11.1 years (median, 1.2; mean, 1.7 ± 1.8 years). Survival length from the time of diagnosis ranged from 0.1 to 11.3 years (median, 2.0; mean, 2.7 ± 2.2 years).

Results of immunoelectrophoreses were available in all cases. Serum or urinary protein studies documented a paraprotein in 28 cases.

Morphologic Characteristics and Immunohistologic Studies

The percentage of bone marrow plasma cells ranged from 3.0 to 30% (mean, 10.5 ± 7.0%). Amyloid was identified in the bone marrow biopsy specimens of 20 of the 57 cases (35%) in which the Congo red stain was performed.

Immunoperoxidase studies identified monoclonal bone marrow plasma cells in 40 cases (17 men, 23 women). The percentage of bone marrow plasma cells in the monoclonal cases ranged from 3.0 to 30% (mean, 12.1 ± 6.8%). The bone marrow plasma cells contained lambda light chains in 30 cases and kappa light chains in 10. There was complete concordance between the light chain class of the bone marrow plasma cells and that of the circulating paraprotein in the 22 monoclonal cases in which a paraprotein was detected. Of the patients with monoclonal plasma cells, ages at bone marrow biopsy ranged from 37.2 to 77.9 years (mean, 59.3 ± 9.7 years). The duration of disease ranged from 0.1 to 4.5 years (median, 0.4; mean, 0.8 ± 1.0 years). Survival length from the time of bone marrow biopsy ranged from 0.0 to 11.1 years (median, 1.1; mean, 1.6 ± 1.9 years). Survival length from the time of diagnosis ranged from 0.1 to 11.3 years (median, 1.9; mean 2.4 ± 2.1 years).

Immunoperoxidase studies in the remaining 22 cases were interpreted as showing polyclonal findings on the basis of approximately equal numbers of plasma cells staining with each of the light chain reagents. The percentage of bone marrow plasma cells ranged from 3 to 30% (mean, 7.5 ± 6.4%). A circulating paraprotein was detected in six cases in which the bone marrow plasma cells appeared to be polyclonal by immunoperoxidase studies. The percentage of plasma cells in these six cases ranged from 3 to 29% (10% or less in five cases, 29% in the sixth). The polyclonal cases were from 17 men and five women, whose ages at bone marrow biopsy ranged from 36.7 to 81.2 years (mean, 62.5 ± 11.6 years). The duration of the disease at the time of bone marrow biopsy ranged from 0.0 to 5.8 years (median, 0.6; mean, 1.2 ± 1.7 years). Survival length from the time of bone marrow biopsy ranged from 0.2 to 5.4 years (median, 1.7; mean,

2.0 ± 1.5 years). Survival length from the time of diagnosis ranged from 0.1 to 9.0 years (median, 2.8; mean, 3.2 ± 2.3 years).

Statistical Analysis

There were significantly more bone marrow plasma cells among patients whose plasma cells were found to be monoclonal by immunoperoxidase studies ($P = 0.011$). Among the monoclonal cases, there was no significant difference in the percentage of bone marrow plasma cells between patients whose plasma cells demonstrated kappa or lambda light chains ($P = 0.464$). The percentage of plasma cells was also greater among patients with a circulating paraprotein (mean, 12.4 ± 8.3%) than among those without a paraprotein (mean, 8.9 ± 5.1%), although these results were of borderline statistical significance ($P = 0.068$).

The duration of disease at the time of bone marrow biopsy did not correlate with sex ($P = 0.775$), the presence of a paraprotein ($P = 0.689$), the percentage of bone marrow plasma cells ($P = 0.366$), or the clonality of the bone marrow plasma cells ($P = 0.190$). Women had significantly more bone marrow plasma cells (mean, 12.6 ± 7.6%) than did men (mean, 8.7 ± 5.8%) ($P = 0.025$); they also had a higher incidence of monoclonality of the bone marrow plasma cells ($P = 0.008$). In addition, they had a higher incidence of a circulating paraprotein than did men (57% for women, 35% for men), but these results were not statistically significant ($P = 0.085$).

The degree of medullary plasmacytosis was related inversely to patients' survival, both from the time of bone marrow biopsy and the time of diagnosis ($P = 0.033$). Patients with higher percentages of bone marrow plasma cells usually had shorter survival times. At any point in time, the risk of death for a patient with 10% plasma cells in the bone marrow compared with one having 5% plasma cells was 1.25 (95% confidence interval, 1.02–1.52). However, there was no correlation between the clonality of the bone marrow plasma cells and survival ($P = 0.43$). There was also no relationship between survival and the presence or absence of a paraprotein ($P = 0.13$) or between survival and sex ($P = 0.156$). Among the monoclonal cases, the light chain subclass of the medullary plasma cells and of the paraprotein in cases associated with a paraprotein showed no correlation with survival ($P = 0.08$). The P values were identical for both measures of survival when their relationships to these parameters were assessed.

DISCUSSION

The term primary amyloidosis traditionally was used to define a disease in which the systemic deposition o

amyloid occurs in the absence of a predisposing underlying chronic inflammatory disorder.[26] However, in 1971 it was reported that these amyloid fibril deposits were related to portions of immunoglobulin light chains.[14] Subsequently, at the Third International Symposium on Amyloidosis in 1979, the biochemistry of the amyloid fibril became the basis of classification of the various types of amyloidosis.[7,18,27] Primary amyloidosis, now designated AL amyloidosis,[7,18,27] is regarded as part of the spectrum of plasma cell dyscrasias with an associated derangement in the synthesis of immunoglobulins.[11,15–17,19,21,28–32] Portions of immunoglobulin light chains, most commonly the fragments that constitute the variable region of the N terminal end of the lambda light chain, are the major constituents of the amyloid fibrillar substance in AL amyloidosis.[1,4,5,8–14,33] Lambda light chains contain six variable region subgroups.[34] In contrast, the amyloid fibrils in secondary amyloidosis, termed AA (amyloid of AA orgin) amyloidosis, are related to a nonimmunoglobulin serum protein.[12] Usually, the survival length in patients with AL amyloidosis is short,[1,27,32,35,36] and previous studies have suggested that congestive heart failure and hepatomegaly may be poor prognostic factors.[20,37]

We used immunoperoxidase stains for immunoglobulin light chains to help identify and quantitate plasma cells in the bone marrow biopsy specimens of 62 patients with AL amyloidosis and to assess the clonality of the plasma cells. This study confirmed our previous finding that the bone marrow plasmacytosis in AL amyloidosis may be monoclonal or polyclonal. Monoclonal plasma cells were identified in 40 cases, with complete concordance between their light chain class and that of the circulating paraprotein in the 22 cases in which a paraprotein was detected. Polyclonal plasma cells were identified in the remaining 22 cases, although a paraprotein was detected in 6 of these polyclonal cases. The percentage of bone marrow plasma cells was significantly higher in patients with monoclonal plasma cells.

Our findings indicate that the quantitation of the bone marrow plasma cells in AL amyloidosis may be of prognostic significance. However, the clonality of the bone marrow plasma cells (monoclonal vs. polyclonal) did not appear to predict survival from the time of bone marrow biopsy or from the time of diagnosis. Although it might be hypothesized that the percentage of bone marrow plasma cells reflects the duration of disease, we found that duration of disease at the time of bone marrow biopsy showed no correlation with the degree of medullary plasmacytosis.

It also might be suggested that the clonality of the bone marrow plasma cells reflects the duration of disease, with a neoplastic clone emerging as the disease progresses. Our findings indicate, however, that the duration of disease does not correlate with the clonality of the bone marrow plasma cells or with the presence or absence of a paraprotein.

It is interesting that women had significantly higher percentages of bone marrow plasma cells than did men. This suggests that women with AL amyloidosis may have a worse clinical course than men. Women also had a higher incidence of monoclonality of their plasma cells.

For several reasons, bone marrow biopsy should be part of the workup of patients with amyloidosis. It primarily should be performed to rule out clinically unsuspected multiple myeloma.[18] In a patient with AL amyloidosis, the diagnosis of multiple myeloma is difficult and sometimes arbitrary because the two syndromes have overlapping manifestations. Therefore, we used clinicopathologic correlations to exclude patients with multiple myeloma. In most cases, one cannot rely only on bone marrow morphologic characteristics and immunostaining to diagnose myeloma in a patient with AL amyloidosis because these patients can have a monoclonal plasmacytosis that does not progress to clinically overt myeloma. However, we did find four cases in which multiple myeloma was diagnosed on the bone marrow biopsy specimen on the basis of sheets of anaplastic plasma cells replacing the marrow space.

Additionally, amyloid was identified in the bone marrow in 20 of our 62 cases, indicating that bone marrow biopsy may be a useful diagnostic procedure in documenting the presence of amyloidosis.[17,18,26,38,39] Immunoperoxidase stains for immunoglobulin light chains should be performed for patients with AL amyloidosis because these stains help one to recognize and quantitate plasma cells in bone marrow biopsy specimens. Moreover, our findings indicate that the quantitation of the medullary plasma cells in AL amyloidosis may be of prognostic significance.

The clinical implications of the clonality of bone marrow plasma cells in AL amyloids remain unclear. This study confirms that the bone marrow plasma cells in this disorder may be monoclonal or polyclonal. However, the presence of a paraprotein in six cases in which the plasma cells appeared to be polyclonal suggests that there may have been a monoclonal plasmacytosis that was not detected because of sampling error or that a reactive polyclonal plasmacytosis, perhaps secondary to infection, may have masked the primary plasma cell clone.[21] These findings suggest that more sensitive techniques—particularly studies for immunoglobulin gene rearrangements—should be used in a prospective study to determine whether a clone of neoplastic plasma cells actually is present in AL amyloidosis cases in which the immunoperoxidase studies appear to indicate a polyclonal plasmacytosis.

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The Expression of CD22 (Leu 14) and CD11c (LeuM5) in Chronic Lymphoproliferative Disorders Using Two-Color Flow Cytometric Analysis

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The monoclonal antibodies (MoAbs) CD22 and CD11c recognize B-lymphocyte- and monocyte-associated antigens, respectively. Reports indicate that when these two MoAbs co-express, they represent a unique marker for hairy cell leukemia (HCL) although neither is specific for that disease. The authors evaluated the expression and diagnostic utility of CD22 and CD11C in specimens from 26 normal subjects, 29 patients with various nonlymphoproliferative disorders (NLPDs), and 75 patients with different types of chronic lymphoproliferative disorders (CLDs) using two-color flow cytometric analysis of peripheral blood lymphocytes. Lymphocytes co-expressed CD22 and CD11c in ≤3% of the normal subjects and in ≤6% of the patients with NLPDs. These markers were expressed in >10% of the lymphocytes of 46% (32/69) of the patients with B-cell CLDs: B-cell chronic-lymphocytic leukemia, 9/41; B-cell non-Hodgkin's lymphoma, 8/14; HCL, 11/11; B-cell lymphoproliferative disorder (NOS), 1/2; and B-cell prolymphocytic leukemia, 1/1. None (0/6) of the lymphocytes of patients with T-cell CLDs expressed >10% CD22-positive (CD22^{+}) or CD11c-positive (CD11c^{+}) cells. The HCL cases demonstrated a unique CD22^{+}CD11c^{+} fluorescence histogram pattern, distinct from other lymphoproliferative disorders, that was characterized by uniformly intense CD11c and CD22 fluorescence. Differences in the expression of the CD22^{+}CD11c^{-} and CD22^{-}CD11c^{+} phenotypes between diagnostic groups were found, most notable was a paucity of CD22^{+}CD11c^{-} cells in lymphocytes of patients with HCL. CD22 also had more variable expression than CD19 and HLA-DR in the cases of B-cell CLD. This study demonstrates that the CD22^{+}CD11c^{+} phenotype is not unique to HCL but is a consistent feature of that disorder and that the immunofluorescence pattern of co-expression in HCL is diagnostically useful. (Key words: CD22; CD11c; Immunophenotyping; Chronic lymphoproliferative disorders; Hairy cell leukemia; Integrin receptors; Monoclonal antibodies) Am J Clin Pathol 1991;96:100–108

Chronic lymphoproliferative disorders (CLDs) are immunophenotypically heterogeneous and include chronic lymphocytic leukemia, prolymphocytic leukemia, hairy cell leukemia (HCL), adult T-cell leukemia/lymphoma, chronic T-γ lymphoproliferative disease, and non-Hodgkin's lymphoma. The lymphoid cells in each of these entities express an array of surface antigens that are helpful in differentiating between these disorders, especially when they are considered in conjunction with the Wright-stain morphology and the clinical history. Unfortunately, no antigenic markers can identify specific CLD subtypes. However, certain lymphoid phenotypes are associated more frequently with specific CLDs, such as the co-expression of CD19 and CD5 in chronic lymphocytic leukemia (CLL),[1,2] the co-expression of CD3 and CD4 in conjunction with the deletions of CD7 and Leu 8 by Sézary cells of mycosis fungoides,[3,4] and the expression of CD8, CD16, CD56, CD57, and Leu 7 (HNK-1) by the large granular lymphocytes of chronic T-γ lymphoproliferative disease.[5-7]

Two monoclonal antibodies (MoAbs), anti-Leu M5 (S-HCL-3; CD11c) and anti-Leu 14 (S-HCL-1; CD22), have been developed to aid in the diagnosis of HCL using HCL cells as the antigenic stimulus.[8] The CD11c antigen has been characterized as an integrin receptor or adherence molecule expressed by monocytes/macrophages, granu-

locytes, and a variety of T- and B-cell lymphoproliferative disorders.[9,10] CD22 is a pan–B-cell antigen found in high concentration on HCL cells.[8] The expression of both antigens by the lymphoid cells of a CLD initially was considered to be specific for HCL.[8] However, we recently reported the results of a preliminary study in which these antigens were co-expressed by the lymphocytes of other CLDs.[11] To define further the diagnostic utility and specificity of these markers, we examined additional individuals, including normal volunteers and patients with a variety of CLDs and nonlymphoproliferative disorders (NLPDs).

METHODS AND MATERIALS

Source of Patient Specimens

Blood specimens submitted to the Department of Laboratory Hematology at The Cleveland Clinic Foundation between June 1, 1987 and September 1, 1990, for immunotyping of CLDs or for lymphocyte-subset analysis of NLPDs were selected for this study. The specimens included 75 from patients with CLDs: 41 patients with B-cell chronic lymphocytic leukemias (B-CLLs); 2 with unclassifiable B-cell lymphoproliferative diseases (B-LPD); 14, circulating B-cell non-Hodgkin's lymphomas (B-NHL); 11, HCL; 1, B-cell prolymphocytic leukemia (B-PLL); 1, T-cell prolymphocytic leukemia (T-PLL); 2, T-cell chronic lymphocytic leukemias (T-CLL); 2, Tγ chronic lymphoproliferative disease (Tγ-LPD); and 1 with Sézary syndrome. Twenty-nine lymphocyte-subset analyses were performed for the following NLPDs: uveitis, viral/reactive lymphocytosis, and interferon therapy; there was no clinical evidence of lymphoid neoplasia, and no clonal proliferation was identified by subset analysis.

A clinical diagnosis was received with each specimen, and a white blood cell (WBC) count with differential and Wright-stained blood smear was examined in each case. An interpretation of the immunophenotypic profile was rendered and each case subclassified after correlation with the WBC count, absolute lymphocyte count, lymphocyte morphology, clinical history, and tissue biopsy diagnosis (bone marrow and lymph node), if performed. All lymphomas where confirmed by lymph node biopsies. A diagnosis of unclassifiable LPD was made when the disease process could not be diagnosed based on the laboratory and clinical parameters examined. The HCL cases included types I and type II, all confirmed by tartrate-resistant, acid-phosphatase staining.

To establish a normal range for the antigens studied, lymphocyte subset analysis was performed on 26 healthy volunteers (laboratory personnel) who had not been ill or received medications for two weeks prior to the subset analysis.

Blood Collection

Whole blood was collected by venipuncture into heparinized or EDTA vacutainer tubes (Becton Dickinson, Rutherford, NJ). A complete blood count with leukocyte differential was performed, and lymphocyte morphology was examined on Wright-stained blood smears in each case. The quantity of blood collected varied depending on the absolute number of lymphoid cells present. The specimens were stored at room temperature for no longer than four hours before processing. If processing was delayed, the whole blood or ficolled cells were placed in RPMI 1640 tissue culture media at room temperature.

Isolation of Mononuclear Cells

A mononuclear cell (MC) suspension was prepared from the whole blood specimen using Ficoll-Paque (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) density-gradient separation. After isolation of the MCs, the cells were washed twice with Hank's balanced salt solution without calcium or magnesium and resuspended in 1.0 mL of modified RPMI 1640. The total cell count was adjusted to $2.5–10.0 \times 10^6$ cells/mL. A leukocyte differential was performed on a Wright-stained cytospin preparation to assess lymphocyte morphology and monocyte/granulocyte contamination.

Monoclonal Antibodies

The MoAbs were obtained from Becton Dickinson Immunology (Mountain View, CA). Their specificity is summarized in Table 1. All of the MoAbs were conjugated with FITC or phycoerythrin (PE) and are commercially available, with the exception of CD11c (Leu M5), which was custom conjugated with PE specifically for this study, courtesy of Becton Dickinson. Conjugated nonimmune

TABLE 1. MONOCLONAL ANTIBODY SPECIFICITIES

Antibody	Specificity
CD3 (Leu 4)	Mature T cells
CD5 (Leu 1)	Pan T cell, B CLL, NHL*
CD11c (Leu M5)	Monocytes/macrophages, NK cells, HCL
CD14 Leu (M3)	Monocytes/macrophages
CD19 (Leu 12)	Pan B cell, including pre B and pre-pre-B cells
CD22 (Leu 14)	Pan B cell
CD45 (HLe-1)	Pan leukocyte
HLA-DR	B cells, activated T cells, monocytes/macrophages

All antibodies were obtained from the Becton-Dickinson Monoclonal Center Mountain View, California. CD11c initially was custom conjugated with phycoerythrin for this evaluation, courtesy of Becton-Dickinson.

* Some low-grade, B-cell non-Hodgkin's lymphomas may express CD5 (*i e.* small lymphocytic and intermediate cell types). See further sources: Burns BF, Warnke RA, Doggett EH, Rouse RV Expression of a T-cell antigen (Leu-1) by B-cell lymphomas. Am J Pathol 1983;113:165—171. and Weisenburger DD, Sanger WG, Armitage JO, Purtilo DT. Intermediate lymphocytic lymphoma immunophenotypic and cytogenetic findings. Blood 1987;69:1617—1621.

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TABLE 2. ANTIBODY COMBINATIONS COMPOSING THE DUAL LABEL IMMUNOTYPING PANELS*

FITC Conjugates	PE Conjugates
IgG₁ (control)	IgG₂ₐ (Control)
HLe-1 (CD45)	Leu M3 (CD14)
Leu 12 (CD19)	Leu 1 (CD5)
Leu 3a (CD4)	Leu 8
Leu 4 (CD3)	HLA-DR
Leu 2 (CD8)	Leu 15 (CD11b)
Leu 16 (CD20)	CRII (CD21)
Leu 4 (CD3)	Leu 15 (CD11b)
IgG₂ₐ (Control)	IgG₂ₐ (Control)
Leu 14 (CD22)	Leu M5 (CD11c)
Leu 9 (CD7)	Leu 1 (CD5)
IgG₁ (Control)	IgG₁ (Control)
Leu 3a (CD4)	Leu 2 (CD8)
Leu 11a (CD16)	Leu 19 (CD56)

FITC = fluorescein isothiocyanate; PE = phycoerythrin.
* Only the pertinent antibody combinations were reported in this study; the actual panels provided a more extensive immunophenotypic profile.

isotype-specific IgG antisera were run simultaneously as negative controls. Panels of MoAbs were constructed to evaluate either the LPDs or the lymphocyte subsets. The antibody combinations composing each of these panels were selected from those shown in Table 2.

Two-Color Direct Immunofluorescence Labeling

Aliquots of 1.0–0.25 × 10⁶ MCs in 100 µL of modified RPMI 1640 were pipetted into each analysis tube. The MCs were vortexed, and 5 µL of mouse IgG-blocking serum (Becton Dickinson Immunology) was added. The mixture was vortexed and incubated at 4 °C for 5 minutes, and 20 µL of the appropriate FITC and PE conjugated MoAbs were added at an optimal dilution, that was predetermined for each antibody. The mixtures were incubated at 4 °C in the dark for 30 minutes. The tubes were vortexed initially and every 10 minutes during the incubation. After incubation, the cells were washed twice in 2.0 mL of cold wash solution (92.3 g hemagglutination buffer and 1.0 g sodium azide) and centrifuged at 300 *g* for 5 minutes at 4 °C. After the final wash, the cells were resuspended in 5 µL of 2.0% paraformaldehyde, and the tubes were covered and stored at 4 °C pending analysis of the specimens.

Flow Cytometric Analysis

MoAb-labeled MCs were analyzed using a FACScan flow cytometer (Becton Dickinson Immunocytometry, San Jose, CA). Gain on fluorescence detectors was adjusted to compensate for spectral overlap of red and green fluorescence signals by using cells co-labeled with MoAbs that had distinctly different antigenic specificity (*i.e.*, CD4-

FITC/CD8-PE). Gated analysis was performed using a dual parameter light-scatter histogram in which a rectilinear gate was placed around the lymphocyte population to be analyzed. The purity of the gated lymphoid cell population was checked using CD45 and CD14 (Leukogate, Becton Dickinson Immunology). Monocyte contamination did not exceed 2%. Quadrant analysis of two-color dot-plot or contour-plot histograms was performed by setting the vertical and horizontal cursors on the isotypic negative-control population so there were <1% positive cells. The true percentage of cells positive for each marker in quadrants 1, 2, and 4 was calculated by subtracting background or nonspecific fluorescence in those quadrants of the isotypic-negative controls.

Statistical Analysis

One hundred twenty-one patients and normal individuals were characterized as belonging to one of five diagnostic groups (B-CLL, B-NHL, HCL, NLPD, and normal) to determine if there were any statistical differences between the groups regarding the percentage of lymphoid cells for each of six phenotypic categories (CD22⁺CD11c⁺, CD22⁺CD11c⁻, CD22⁻CD11c⁺, CD22⁺, CD19⁺, and HLA-DR⁺). Because the percentages of lymphoid cells within the phenotypic categories were not normally distributed, the nonparametric Kruskal-Wallis test was used to compare the diagnostic groups within each of the six categories. Dunn's procedure for multiple comparisons was used if the tests showed significant differences between groups. Total CD19⁺ and CD22⁺ cells were compared to the normal group using the Wilcoxon's rank sum test. Differences were considered significant for *P* values ≤ 0.05. The cases of B-LPD (2), B-PLL (1) and T-cell disorders (6) were not included in the statistical analysis because of the small number of cases or the paucity of positive cells in the phenotypes studied (T-cell cases).

RESULTS

The hematologic data and immunophenotypic results for each diagnostic group are summarized in Table 3.

Normal Subjects and Patients with Nonlymphoproliferative Disorders

A normal range of expression was established for the CD22 and CD11c antigens using 26 normal subjects. Only a small number of CD22⁺CD11c⁺ cells were detectable (median 2%, range 0–3%), whereas larger populations of CD22⁺CD11c⁻ cells (median 7%, range 3–15%) and CD22⁻CD11c⁺ cells (median 11%, range 3–22%) were found.

Two-Color Flow Cytometric Analysis

TABLE 3. HEMATOLOGIC DATA AND IMMUNOPHENOTYPIC RESULTS FOR EACH DIAGNOSTIC GROUP

Diagnostic Group	WBC $(\times 10^9/L)$	Lymphocytes $(\times 10^9/L)$	CD22+ CD11c+ (%)	CD22+ CD11c− (%)	CD22− CD11c+ (%)
Normals					
n	26	26	26	26	26
Median	6	2	2	7	11
Range	4–13	1–3	0–3	3–15	3–22
NLPD					
n	29	29	29	29	29
Median	8	2	1	7	5
Range	4–14	1–8	1–6	1–21	1–33
B-CLL					
n	41	41	41	39	40
Median	28	25	5	13	4
Range	7–238	5–236	0–87	1–56	1–54
B-NHL					
n	14	14	14	14	14
Median	18	11	13	32	4
Range	7–95	2–92	0–44	0–72	0–48
HCL					
n	11	11	11	11	11
Median	13	8	55	1	7
Range	1–188	1–182	14–97	0–20	1–16
B-LPD					
n	2	2	2	2	2
Median	38	17	37	26	4
Range	17–58	11–23	2–71	0–51	2–6
B-PLL					
n	1	1	1	1	1
Median	66	62	41	5	35
Range	66	62	41	5	35

Results of the 29 NLPD cases were similar to those of the normal group in that there was a paucity of CD22+CD11c+ cells (median 1%, range 1–6%) and greater numbers of CD22+CD11c− cells (median 7%, range 1–21%) and CD22−CD11c+ cells (median 5%, range 1–33%). The only significant difference between the results of the normal group and NLPDs group was found in the CD22−CD11c+ phenotype, which was expressed to a greater extent in the normals.

There was no statistically significant difference between the normal and NLPD groups for any of the phenotypic categories studied.

B-Cell Lymphoproliferative Disorders

CD22+CD11c+ Phenotype. Figure 1 demonstrates the median percentage of cells that labeled for CD22 and CD11c in each of the groups studied. Among the major lymphoproliferative disorder groups, the HCL cases had the largest median percentage of CD22+CD11c+ cells (55%), followed by the B-NHL (13%) and B-CLL (5%) groups. The difference between the median number of CD22+CD11c+ cells in the normal group and the B-cell neoplastic groups was significant only for the HCL group ($P < 0.05$); in contrast, the NLPD group had a significantly lower median than the HCL, B-NHL, and B-CLL groups

($P < 0.05$). In addition, the high median percentage of CD22+CD11c+ cells observed in the HCL group when compared with the B-CLL group was significant ($P < 0.05$). This was not true for the B-NHL group. The two
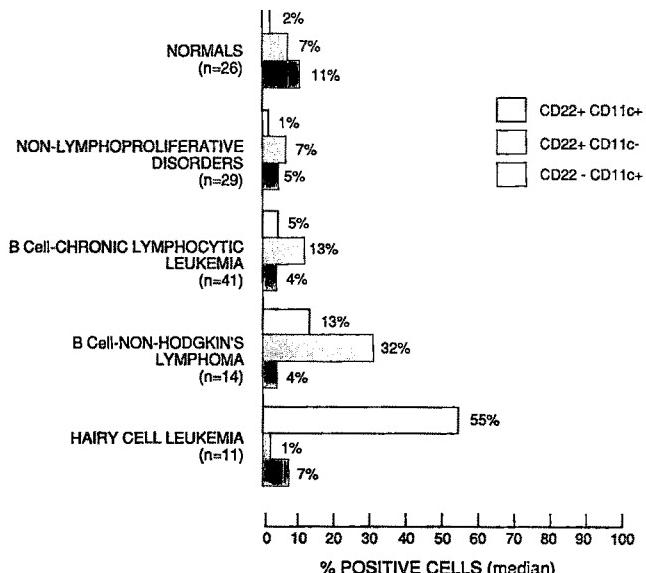
FIG. 1. The distribution of CD22/CD11c immunotypes (medians) within each study group.

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B-LPD cases exhibited the extremes of expression for this phenotype (2% and 71%), and 41% of the cells of the B-PLL case co-expressed these markers.

Although the median percentage of CD22$^+$CD11c$^+$ cells was significantly lower in the B-CLL and B-NHL groups than in the HCL group, there were many cases in which this percentage of dual-labeled cells exceeded 10%. In fact, specimens from 32 (46%) of the 69 patients with B-CLDs co-expressed CD22 and CD11c in >10% of the MCs: B-CLL, 22% (9/41); B-NHL, 57% (8/14); and HCL, 100% (11/11) (Table 4). The distribution of cases according to the percentage of CD22$^+$CD11c$^+$ cells in the total cell population analyzed and as a percentage of total B cells (CD19$^+$) is illustrated in Figure 2. A similar distribution of cases was observed for both cell populations. However, more cases had higher percentages of CD22$^+$CD11c$^+$ cells when represented as a percentage of total B cells.

CD22$^+$CD11c$^-$ Phenotype. The percentage of CD22$^+$CD11c$^-$ cells was variable among cases within the different diagnostic groups. The median number of CD22$^+$CD11c$^-$ cells was in excess of the CD22$^+$CD11c$^+$ cells in the B-CLL and B-NHL groups. In contrast, specimens from the HCL and B-PLL cases had a striking paucity of cells bearing this phenotype (median 1%, 11 cases ≤5% and 1 case 20%) with a preponderance of CD22$^+$CD11c$^+$ cells. The median of the HCL group (1%) was significantly lower (P < 0.05) than the medians of the B-NHL (32%) and B-CLL (13%) groups. In addition, the median percentage of CD22$^+$CD11c$^-$ cells in B-NHL groups was significantly greater than that found in the normal (7%) and NLPD (7%) groups (P < 0.05). The two B-LPD cases demonstrated 0% and 51% CD22$^+$CD11c$^-$ cells, which inversely correlated with the percentage of CD22$^+$CD11c$^+$ cells (71% and 2%).

CD22$^-$CD11c$^+$ Phenotype. In general, the CD22$^-$CD11c$^+$ cell populations in the B-CLDs were small and similar to those of the normal and NLPD groups. The median percentages of all B-CLD groups was: 4% B-CLL; 4% B-NHL; 7% HCL; 4% B-LPD; and 35% B-PLL (1 case). Only the normal and B-CLL groups were statistically different (P < 0.05). There was variable expression of this phenotype among cases within the different groups. The

TABLE 4. SUMMARY OF CASES WITH >10% CD22$^+$CD11c$^+$ LYMPHOID CELLS

Normal subjects	0/26
Nonlymphoproliferative disorders	0/29
T-cell lymphoproliferative disorders	0/6
B-cell lymphoproliferative disorders	32/69
Chronic lymphocytic leukemia	9/41
Lymphoproliferative disorder (NOS)	1/2
Non-Hodgkin's lymphoma	8/14
Hairy cell leukemia	11/11
Prolymphocytic leukemia	1/1
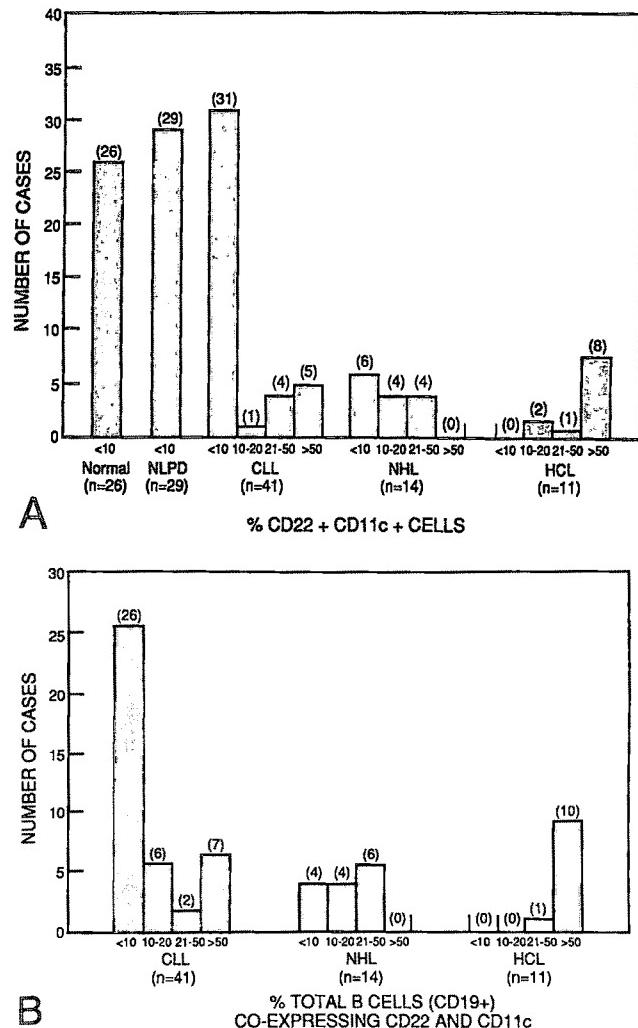
FIG. 2. The distribution of cases within each study group based on (*A*) the percent of the CD22$^+$CD11c$^+$ cells in the total cell population analyzed and (*B*) the percent of total B-cells (CD19$^+$).

number of cases and the range of cells in each group with >10% CD22$^-$CD11c$^+$ cells was: 10 B-CLL (12–54%); 4 B-NHL(11–48%); 2 HCL(14% and 16%); and 1 B-PLL(35%).

Comparison of Total CD22$^+$, CD19$^+$, and HLA-DR$^+$ (CD3$^-$) Populations. CD22 was compared with CD19 and HLA-DR as a B-cell marker in the B-CLD groups (Table 5). There was no significant difference in the median percentage of CD22$^+$, CD19$^+$, and HLA-DR$^+$ cells between the CLL, NHL, and HCL groups when each marker was independently evaluated. However, differences were noted in the expression of these markers within diagnostic groups. A statistically significant difference (P < 0.001) was observed in the B-CLL group when CD19 (77%) and HLA-DR (80%) were compared with CD22 (24%). A borderline significant difference (P = 0.05) between these markers for the B-NHL group (76%, 75%, and 46%) also was observed. In contrast, good concordance of these im-

TABLE 5. COMPARISON OF THE TOTAL CD22⁺, CD19⁺,
AND HLA-DR⁺ CELL POPULATIONS (MEDIANS) IN THE
B-CELL LYMPHOPROLIFERATIVE DISORDERS

	Total CD22+	Total CD19+	HLA-DR+ (CD3⁻)
B-CLL* (n = 41)	23%	77%	80%
B-NHL† (n = 14)	46%	76%	75%
HCL (n = 11)	60%	66%	71%
B-LPD (n = 2)	62%	35%	66%
B-PLL (n = 1)	46%	91%	79%

* $P < 0.001$.
† $P = 0.05$.

munotypes was noted among the HCL cases (66%, 71%, and 60%). The total HLA-DR⁺ (CD3⁻) cell population correlated more often with CD19 than with CD22. There was good correlation between all three B-cell markers in one of the B-LPD cases (53% CD22⁺, 59% CD19⁺, and 63% HLA-DR⁺) but discordant values were found in the other case (71% CD22⁺, 11% CD19⁺, and 68% HLA-DR⁺). CD19 and HLA-DR were expressed to a greater extent (91% and 79%) than CD22 (46%) in the one case of B-PLL studied.

Fluorescence Patterns of CD22/CD11c Expression. The B-CLD groups demonstrated four basic fluorescence histogram patterns of CD22/CD11c antigen co-expression. The most common pattern observed in the B-CLL, B-NHL, B-LPD, and B-PLL cases was weak CD22 expression, with a graded pattern of CD11c expression, extending upward from the lower left side of quadrant 2 as shown in Figure 3A. The fluorescence patterns in Figures 3B and C were seen less frequently. All HCL cases exhibited the same fluorescence pattern, which was characterized by strong CD11c and moderate CD22 expression with a well-defined population of co-labeling cells located in the upper central portion of quadrant 2 (Fig. 4). A similar pattern was observed in only one non-HCL case, a B-LPD (Fig. 5).

There was no definable fluorescence pattern for the lymphocytes co-expressing these markers in the normal and NLPD groups, or in the T-cell CLD group. The positive cells were few in number and randomly dispersed in the lower portion of the second quadrant.

T-Cell Lymphoproliferative Disorders

The six T-cell processes (T-CLL, Tγ-LPD [2], T-PLL, T-LPD [NOS]) and Sézary syndrome had small numbers of CD22⁺CD11c⁺ cells (range 0–3%). The populations expressing the CD22⁺CD11c⁻ and CD22⁻CD11c⁺ phenotypes also were small (0–1%) for the T-CLL, T-PLL, T-LPD, and Sézary syndrome cases, but the two Tγ-LPD had more variability for these two phenotypes 4% and 16%, and 1% and 7%, respectively.

DISCUSSION

We previously reported the results of a preliminary study in which CD11c and CD22 were co-expressed in chronic lymphoid leukemias other than HCL.[11] The current study has evaluated further the expression of CD22 and CD11c in patients with a variety of CLDs and NLPDs and in normal subjects in an effort to better define the diagnostic utility and specificity of the two MoAbs. The MoAbs were produced by hybridomas developed by immunizing CD-1 mice with HCL cells in an attempt to find a specific marker for that disease. It was found that anti-CD22 exhibited pan–B-cell reactivity, anti-CD11c demonstrated monocyte/macrophage and granulocyte specificity, and both showed reactivity with HCL cells. Initial studies reported by Schwarting and colleagues[8] indicated that when these antibodies were co-expressed, they were specific for HCL. This finding was supported in part by Kristensen and co-workers,[12] who evaluated unconjugated anti-CD11c and anti-CD20 using two-color flow cytometry for the detection of hairy cells. In contrast, Inghirami and colleagues[9] found CD11c to be expressed in several different B-cell neoplasms and mycosis fungoides, although no co-labeling B-cell marker was studied.

Our findings indicate that CD22 and CD11c are co-expressed in only a small percentage of lymphocytes in normal subjects (also found by Schwarting),[8] in patients with NLPD, and in patients with T-cell lymphoproliferative diseases, but are commonly co-expressed in >10% of the lymphocytes in a variety of B-CLDs. The frequency and extent of co-expression among cases were variable and similar to the reported expression of CD11c in lymphoid neoplasms by Inghirami and colleagues[9] with the exception that mycosis fungoides did not express this antigen in our study. One hundred percent (11/11) of the HCL cases we studied exhibited these markers, and this group had the largest median number of CD22⁺CD11c⁺ co-labeling cells, which was statistically significant when compared with the other groups, except the B-NHL group. Unfortunately, because of the considerable overlap of the percentage of CD22⁺CD11c⁺ cells among cases between the different B-CLD groups (Fig. 2), this parameter (% CD22⁺CD11c⁺) is nonspecific and cannot be used to distinguish HCL from other B-CLDs. However, the HCL cases demonstrated a distinctive flow cytometric pattern of fluorescence in which the CD22⁺CD11c⁺ cells were located in the mid- to upper-central portion of the second quadrant of the histogram in a well-defined group (Fig. 4). This was indicative of uniformly higher CD22 and CD11c antigen density, a characteristic feature of HCL compared with other B-CLDs, and should be diagnostically useful. The stronger expression of these antigens in HCL also has been noted by others.[8,12] In contrast, when

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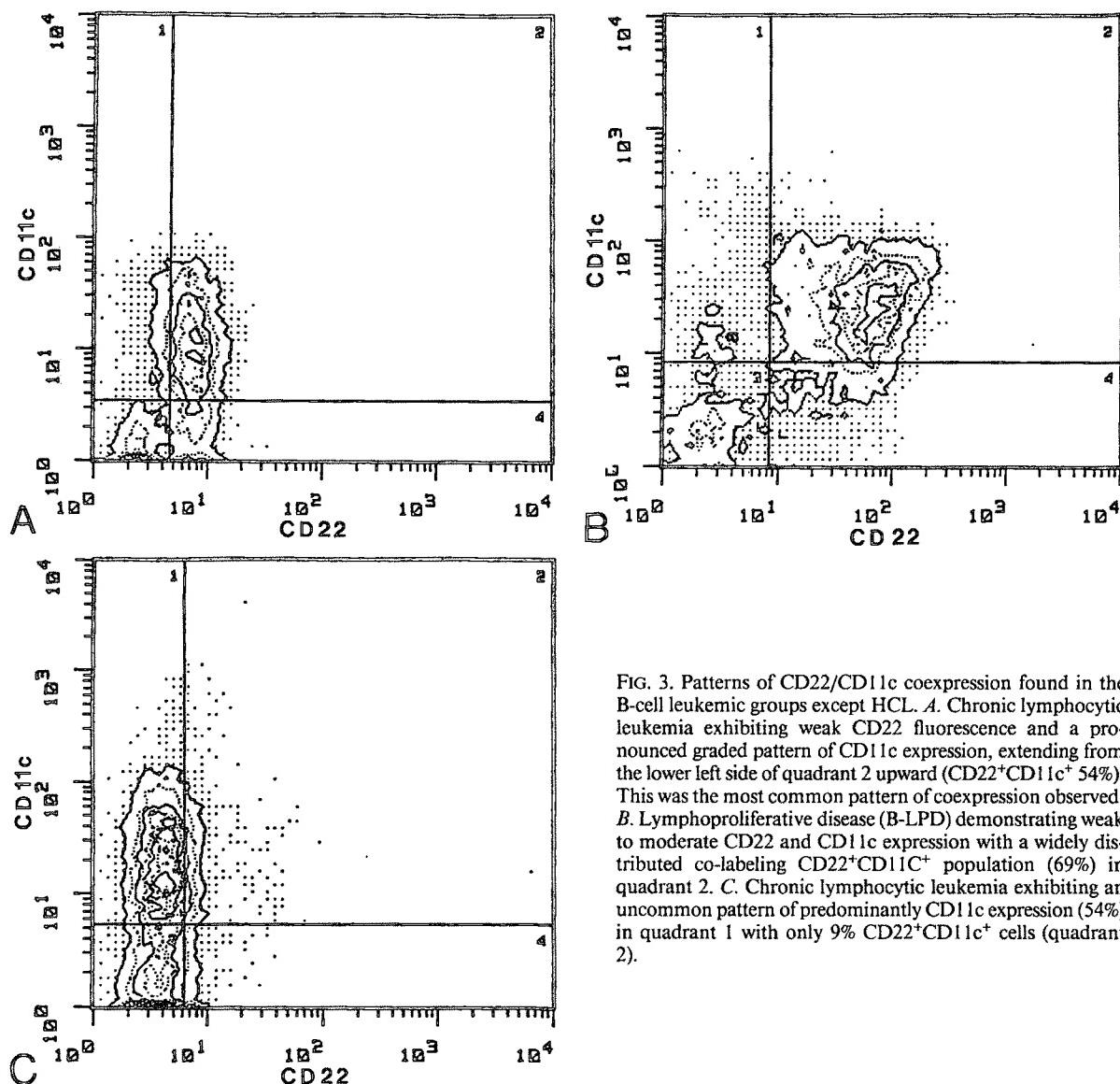
FIG. 3. Patterns of CD22/CD11c coexpression found in the B-cell leukemic groups except HCL. *A.* Chronic lymphocytic leukemia exhibiting weak CD22 fluorescence and a pronounced graded pattern of CD11c expression, extending from the lower left side of quadrant 2 upward (CD22$^+$CD11c$^+$ 54%). This was the most common pattern of coexpression observed. *B.* Lymphoproliferative disease (B-LPD) demonstrating weak to moderate CD22 and CD11c expression with a widely distributed co-labeling CD22$^+$CD11C$^+$ population (69%) in quadrant 2. *C.* Chronic lymphocytic leukemia exhibiting an uncommon pattern of predominantly CD11c expression (54%) in quadrant 1 with only 9% CD22$^+$CD11c$^+$ cells (quadrant 2).

CD22 and CD11c were co-expressed by the neoplastic cells in B-CLL, B-NHL, B-LPD, and B-PLL CD11c expression generally was weaker, with a wide range of fluorescence intensity (Fig. 3). Another finding that may be useful in distinguishing HCL from other B-CLDs is that there is a paucity of CD22$^+$CD11c$^-$ cells in HCL but a significant number of neoplastic cells with this phenotype in the other B-CLDs.

The only exception to the fluorescent patterns observed in the non-HCL diseases was one of the B-LPD cases that had 71% CD22$^+$CD11c$^+$ cells with a fluorescence pattern similar to that seen in the HCL cases. This patient was a 60-year-old woman who presented with fatigue and splenomegaly and a WBC count of 58 × 10^9/L with 80% immature lymphoid cells but no lymphadenopathy. The bone marrow had a diffuse infiltrative pattern of involve-

ment. Morphologically the cells were intermediate between those of PLL and HCL, having round to oval nuclei and coarsely granular to clumped chromatin, small nucleoli, and moderate amounts of light blue cytoplasm. Cytochemically the cells demonstrated tartrate-sensitive, acid-phosphatase activity. The fluorescence pattern was similar to that of HCL (Fig. 5) but had less intense fluorescence for both markers, and the overall pattern of the contour plot was not as well defined. A diagnosis of B-LPD was rendered because of the unusual clinical history, atypical cell morphology, and the absence of tartrate-resistant acid-phosphatase activity and ultrastructural features that would support the diagnosis of HCL. No other tissue was obtained for diagnostic evaluation, and the patient's clinical condition remains stable after two years.

The other B-LPD case was that of a 76-year-old man

Two-Color Flow Cytometric Analysis
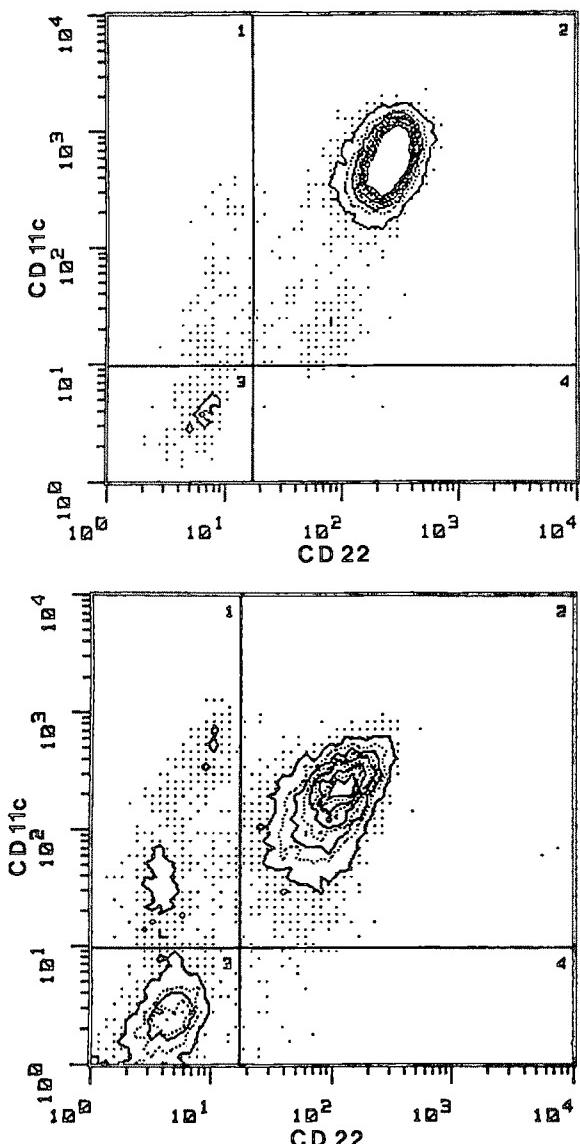
FIG. 4 (*upper*). Hairy cell leukemia exhibiting strong CD11c and moderate CD22 expression with a well-defined population of $CD22^+CD11c^+$ cells (96%) in the upper-central portion of quadrant 2.

FIG. 5 (*lower*). Lymphoproliferative disease (B-LPD) exhibiting a fluorescence pattern similar to HCL but having less intense fluorescence for both antigens and a less well-defined and discrete $CD22^+CD11c^+$ cell population (70%) in quadrant 2.

who presented with fatigue, splenomegaly, and a WBC count of 17 × 10⁹/L with 57% immature lymphoid cells. There was no bone marrow involvement, lymphadenopathy, or hepatomegaly at presentation. The lymphoid cells were morphologically similar to those of the other B-LPD case. Within two years, the patient's WBC count increased to 49 × 10⁹/L with 91% immature lymphoid cells, and he developed hepatomegaly but no lymphadenopathy. A follow-up bone marrow and acid-phosphatase stain was

not done. $CD22^+CD11c^+$ cells comprised 2% of the lymphoid cells, but $CD22^-CD11c^+$ cells comprised 51% of the lymphoid cells. These cases resemble those reported recently by Hanson and colleagues[13] and may represent a CLD resulting from an arrest of lymphoid maturation in a stage of development between a small lymphocyte and the normal hairy cell counterpart in the spleen.

Lymphocytes expressing the $CD22^-CD11c^+$ phenotype were present in variable numbers in all groups, with the specimens of the normal subjects having a median of 11%, and those of the B-CLD group exhibiting 17 cases with >10% (median 16%, range 11–54%). The unusually large number of lymphoid cells expressing this phenotype may be caused by the increased numbers of natural killer cells in some cases. Another possible explanation, especially in the few cases with large numbers of $CD22^-CD11c^+$ cells, would be aberrant antigen expression by the neoplastic cells.

CD22 was found to have more variable expression than CD19 and HLA-DR in the B-cell disorder groups. While CD19 and HLA-DR often were present in a similar percentage of cells, CD22 was consistently expressed in a smaller percentage of cells in all of the B-CLD groups except HCL, for which the three markers were quantitatively similar. However, significant differences were found between the lower median percentage of total $CD22^+$ cells and the $CD19^+$ and $HLA-DR^+$ cells in the CLL, NHL, and PLL cases. The lack of CD22 expression by some leukemic cells in these B-CLDs would warrant caution in the use of CD22 as the only pan–B-cell marker in a panel used to phenotype B-CLDs.

The diagnostic utility of using anti-CD11c in combination with other pan–B-cell antibodies, such as anti-CD19 and -CD20, was not examined. However, Kristensen and colleagues[12] studied the co-expression of CD11c and CD20 using a dual label and found them to be specific for HCL. We have observed two cases in which the results for the CD19 and CD20 antigen expression suggested that using either of these two markers in a dual label could lead to misinterpretation of the data. The first case was one of HCL, for which there was essentially no CD20 expression but CD19 was in agreement with the CD22/CD11c results (72% and 76%, respectively). The second case was one of CLL that was $CD22^-$ but had comparable CD11c and CD19 expression. These anecdotal observations suggest caution is needed when using anti-CD11c and pan–B-cell markers other than CD22 as an adjunctive test in diagnosing HCL.

HCL is a neoplasm of B lymphocytes as determined by the presence of monoclonal surface immunoglobulin and immunoglobulin gene rearrangement studies[14] and the expression of B-cell associated antigens (HLA-DR, CD19, CD20, CD10, and PCA-1).[14–17] HCL cells also ex-

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press the pan-leukocyte antigen CD45, the receptor for interleukin 2 (CD25)[14,15] and, occasionally, myelomonocyte antigens, such as CD11b, CD13, CD14, and CD33.[12] Despite this spectrum of reactivity with MoAbs, none have been found to be specific for HCL. Therefore, these markers have been only indirectly helpful in distinguishing this disease from other CLDs when traditional diagnostic parameters, such as morphology, ultrastructural findings, and cytochemical studies, have been equivocal. This study has demonstrated that, although the co-expression of CD22 and CD11c by neoplastic B cells is not diagnostic of HCL, the unique dual label pattern of intense CD22 and CD11c fluorescence in the majority of B cells in association with few CD22$^+$CD11c$^-$ cells should be useful in distinguishing HCL from other B-CLDs in most instances.

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Original Article

Comparison of an Isoelectric Focusing Technique and High-Performance Liquid Chromatography for Determination of Fetal Hemoglobin Levels

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EARL SHRAGO, M.D.,[3] TERRY L. SPENNETTA,[3] AND GARY G. GIULIAN, B.A.[4]

The authors previously have reported measurements of fetal hemoglobin in infants in blood samples taken at autopsy using an isoelectric focusing (IEF) procedure. The current study was undertaken to compare this methodology with a high-performance liquid chromatography (HPLC) procedure. The correlation coefficient between the IEF and HPLC procedures was 0.938. The HPLC method is technically easier and has fewer disadvantages than the IEF procedure and is recommended for the determination of fetal hemoglobin levels. (Key words: Fetal hemoglobin; High-performance liquid chromatography; Isoelectric focusing) Am J Clin Pathol 1991;96:109–110

We previously have reported[1] percent fetal hemoglobin determinations (%HbF) from infants using an isoelectric focusing (IEF) procedure in blood samples taken at autopsy. The IEF procedure has serious disadvantages. The %HbF measurements may be influenced by the amount of staining of the gel and by leakage of samples from adjacent lanes on the gel. This study was undertaken to compare the IEF method with that of the high-performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Using the HPLC and IEF methods, we measured %HbF in blood samples taken at autopsy from 46 infants (newborn to 2 years). The blood samples were part of a study measuring %HbF in victims of sudden infant death syndrome compared with controls, and many samples were not obtained until several hours after death. The samples

were stored in sterile vacuum tubes containing EDTA at 4 °C for up to two years before being analyzed.

The IEF analyses used a high-voltage, vertical-slab isoelectric focusing procedure to measure hemoglobin subunits using whole blood mixed with a sample buffer and loaded onto a polyacrylamide gel. Details of this method have been described previously.[1,2] The gels were scanned with a densitometer, and the percent fetal hemoglobin was determined by calculating the ratio of the sum of the gamma peaks to the sum of the gamma and beta peaks.

HPLC measurements were made using the reversed-phase HPLC procedure described by Shelton.[3] All blood sample measurements were made on red cell lysates, and some samples had %HbF measurements taken on blood dried on filter paper. The samples were analyzed by HPLC using a Vydac C-4 column (Vydac, Hesperia. CA) and acetonitrile-water-trifluoroacetic acid developers. This procedure takes about 60 minutes to complete the separation of the globin chains, after which the chromatogram and the area under each peak are printed. The percent of fetal hemoglobin was calculated as the sum of the gamma chains, G gamma, A gamma, and A gamma T (*i.e.*, the sum of all the peaks after the alpha peak), divided by the sum of the gamma chains plus the beta and prebeta chains.[4]

The data were plotted, and regression equations calculated by Grapher, version 1.75 (Golden Software, Inc.).

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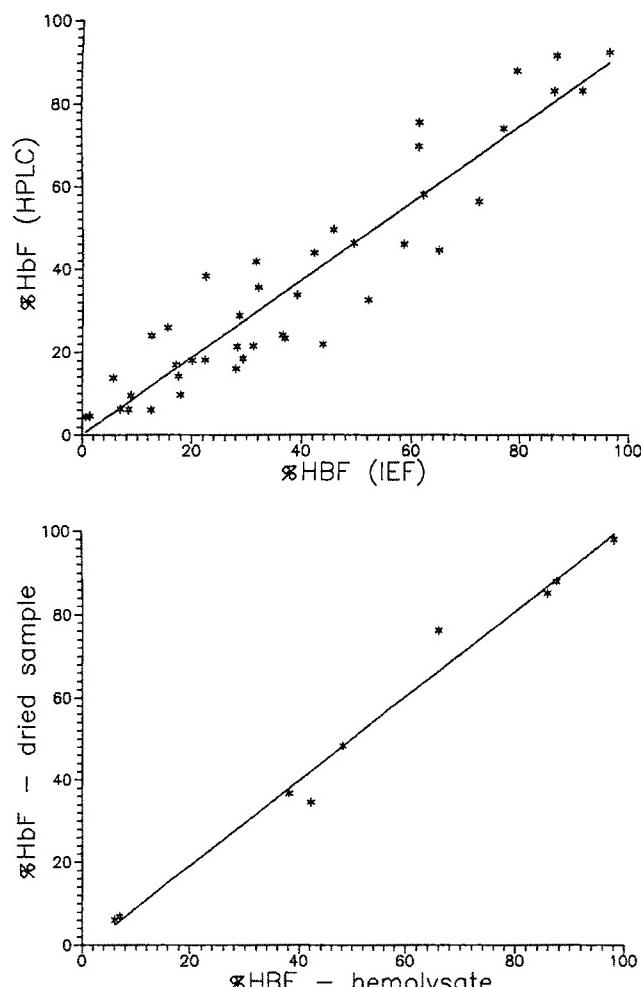
FIG. 1 (*upper*). Scatterplot and line of best fit of fetal hemoglobin levels in 42 blood samples as determined by high-performance liquid chromatography and isoelectric focusing.

FIG. 2 (*lower*). Scatterplot and line of best fit of fetal hemoglobin levels determined by high-performance liquid chromatography in nine blood samples comparing dried samples to hemolysates.

RESULTS

Our results show the HPLC method to give reliable measurements of %HbF. Each sample was replicated at least once. The correlation between replications was 0.98, with an intercept of 0.01, a slope of 0.97, and a standard error of the Y estimate of 0.05. The correlation coefficient between HPLC and IEF %HbF determinations on 42

samples was 0.938. Figure 1 shows a scatterplot and line of best fit of %HbF determined by the HPLC method plotted against %HbF determined by the IEF procedure. The regression equation was IEF %F = 0.045 + 0.948 × (HPLC %F). The standard error of the Y estimate was 0.09. Four samples were excluded from the analysis because their HPLC results contained unidentifiable peaks with poor morphology, and their %HbF as determined by the HPLC method was more than 30% greater than that by the IEF. For these samples the HPLC analysis was conducted several months after the IEF, and we assume that the HPLC results are caused by breakdown or contamination of the blood sample.

There were nine samples from which both hemolysates were made and a few drops of blood were dried on filter paper. The correlation coefficient of HPLC determinations using hemolysates with dried blood samples was 0.99, and the regression equation was filter paper %HbF = −0.01483 + 1.029 × (hemolysate %HbF). The standard error of the Y estimate was 0.05. Figure 2 shows a scatterplot and best fitting line of %HbF obtained from dried samples plotted against %HbF obtained from hemolysates.

DISCUSSION

The HPLC method[3] is accurate for the determination of fetal hemoglobin levels throughout the range of 0–100%. This procedure can be used on blood samples obtained at autopsy and stored dried or refrigerated in vacutainers with EDTA. The HPLC method also avoids some of the disadvantages of the IEF method. In the IEF procedure there can be leakage between lanes on the gel, and the percentage of HbF at the high and low ends of the range can be affected by the amount of staining and destaining of the gel.

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Auer Rod-like Inclusions in Circulating Lymphoma Cells

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Circulating malignant lymphocytes from a 55-year-old woman with small cleaved follicular center cell lymphoma contained azurophilic splinter-shaped cytoplasmic inclusions. By light microscopic and ultrastructural criteria, these structures closely resembled Auer rods found in acute myeloid leukemia; however, the authors could not find cytochemical evidence of lysosomal origin (results were negative for myeloperoxidase, Sudan black B, acid phosphatase, and periodic acid-Schiff). Immunostaining and flow cytometric analysis confirmed a monoclonal IgM-kappa immunophenotype of the circulating malignant lymphoid cells. The inclusions did not show specific immunoglobulin staining by light microscopic or electron microscopic immunostaining techniques. The authors conclude that these membrane-bound inclusions probably represent aberrant lysosomes in the malignant cells. (Key words: Auer rods; Lymphoma; Cytoplasmic inclusions; Lysosomes; Immunoglobulin; Lymphocytes) Am J Clin Pathol 1991;96:111–115

Intracytoplasmic inclusions have been described in circulating malignant lymphocytes of various lymphoproliferative disorders. A variety of azurophilic and basophilic round to oval cytoplasmic granules that ultrastructurally resemble microtubules,[1] lysosomes,[2–4] ribosomal-lamellar complexes,[4,5] viral particles,[4] and mitochondria[6,7] have been reported in acute lymphoblastic leukemia. Large cytoplasmic proteinaceous crystalline structures have been described in chronic lymphocytic leukemia,[8–10] plasma cell tumors,[11–14] and B-cell lymphoma.[15] The cytoplasmic crystals are quite distinct from the splinter-shaped azurophilic Auer rods found in acute myeloid leukemias.[16–19] Needle-like crystals, which morphologically and ultrastructurally resemble Auer rods, have been found in the cytoplasm of neoplastic lymphocytes in single cases of chronic lymphocytic leukemia[20] and prolymphocytic leukemia,[21] and two cases of multiple myeloma.[13,22] We describe a case of small cleaved follicular center cell lymphoma in leukemic phase with splinter-shaped Auer rod–like cytoplasmic inclusions.

REPORT OF A CASE

A 55-year-old Guatemalan woman, with cough, fever, and malaise, was seen in February 1987. A chest roentgenogram showed a right upper lobe infiltrate, and on examination she had diffuse lymphadenopathy in the cervical, inguinal, and axillary regions. A lymph node biopsy was performed. The specimen was interpreted as showing malignant lymphoma, follicular, small cleaved cell type (Working Formulation). Additional workup demonstrated a normal complete blood count, serum chemistries, and protein electrophoresis.

The patient was treated with chlorambucil, vincristine, and prednisone. She did well until January 1989, when she had generalized lymphadenopathy and circulating lymphoma cells. Fine-needle aspiration cytologic examination of inguinal lymph nodes and bone marrow biopsy demonstrated involvement by small cleaved cell lymphoma. Chemotherapy was started and consisted of bleomycin, vincristine, Cytoxan®(Bristol-Meyers/Mead Johnson Oncology, Evansville, IN), Decadron® (Merck Sharp and Dohme, West Point, PA), and Adriamycin® (Adria Laboratories, Dublin, OH).

In June 1989, the patient returned to the clinic with enlarging lymph nodes. Her white blood cell count was 8.7 × 10⁹ cells/L, of which 0.39 were lymphocytes. Many of these cells were identified morphologically as circulating small cleaved lymphoma cells. Intracytoplasmic inclusions resembling Auer rods were reported in a number of these cells.

The patient received a chemotherapeutic regimen of Decadron, cytosine arabinoside, and cisplatin. The number of circulating lymphoma cells decreased, and subsequently fewer inclusion bodies were found. She remains in clinical remission.

MATERIALS AND METHODS

Light Microscopic Preparations

Peripheral blood direct and buffy coat smears were stained with Wright-Giemsa and for the presence of my-

eloperoxidase (MPO), periodic acid-Schiff (PAS), Sudan black B (SBB), and acid phosphatase (ACP).[23-25]

Electron Microscopic Preparations

Peripheral blood cells isolated from the buffy coat of venous blood anticoagulated with tripotassium ethylene-diaminetetraacetic acid (EDTA) were fixed immediately in cold 3% glutaraldehyde in 0.1 mol/L cacodylate buffer (pH 7.4), postfixed in osmium tetroxide, embedded in Epon® (DuPont, Wilmington, DE), sectioned and stained with uranyl acetate and lead citrate, and examined with a Philips EM 301® electron microscope (Philips, Eindhoven, The Netherlands).[26]

Flow Cytometry

Peripheral blood was collected in EDTA. Lymphocyte subset analysis was performed as previously described,[27] except that a Coulter Profile® Flow Cytometer (Coulter Electronics, Hialeah, FL) was used. The following antibodies were used: anti-CD1 (T6), anti-CD2 (T11), anti-CD3 (T3), anti-CD4 (T4), anti-CD5 (T1), anti-CD5 (T1), anti-CD8 (T8), anti-CD10 (common acute lymphoblastic leukemia antigen), anti-CD19 (B4), anti-CD20 (B1), antikappa, and antilambda.

Immunocytology

Peripheral blood buffy coat preparations were stained by an avidin–biotin–peroxidase technique[28] for CD3 (Leu-4), CD22 (Leu-14), IgG, IgM, IgA, kappa, and lambda (prediluted antibodies from DAKO Corporation, Carpinteria, CA).

Immunoelectron Microscopy

The technique of Gourdin and associates[26] was used, with minor modifications, to detect immunoglobulin light chain staining. Peripheral blood cells isolated from the buffy coat of EDTA-anticoagulated venous blood were fixed in 3% glutaraldehyde in 0.1 mol/L cacodylate buffer (pH 7.4) for 30 minutes, washed twice with 0.05 mol/L TRIS buffer (pH 7.6), incubated in 0.5% Triton-X (EM Science, Gibbstown, NJ) for 15 minutes, and washed again with TRIS buffer. After incubation with the appropriate primary antibody for one hour at room temperature, the cells were washed with TRIS buffer, incubated with biotinylated secondary antibody for 30 minutes at room temperature, and washed three times with TRIS buffer. Avidin: biotinylated horseradish peroxidase complex (ABC) was added, incubated for 30 minutes, and washed. The specimen was postfixed and embedded in Epon, as described above.

RESULTS

Light Microscopy

The lymphoma cells were characterized by ovoid to irregularly shaped nuclei, coarse clumped chromatin, and scant to moderate amounts of basophilic cytoplasm. Many cells showed scattered, small, irregular azurophilic granules in the cytoplasm. Occasional cells were larger, with fine chromatin and one to two small nuclei.

Azurophilic, splinter-shaped, variably sized crystals were found in the cytoplasm of the malignant lymphocytes of the peripheral blood (Fig. 1). Approximately 7% of the cells had these inclusions during relapse. When peripheral blood was examined during periods of clinical remission, only rare (<1%) lymphocytes contained crystals. Review of bone marrow aspirate smears and lymph node imprints involved with lymphoma demonstrated no evidence of cytoplasmic inclusions.

Most cells showed single crystals, but an occasional cell contained multiple crystals (Fig. 1). Many crystals overlapped the nucleus. Some had a more rounded shape or appeared beaded; however, most had a splinter shape comparable to that seen in the Auer rods of acute myeloid leukemia. There were crystals lying free next to disrupted cells on the feathered edge of the smear (Fig. 1). Other circulating lymphoma cells were cytologically similar to those containing crystals.

In cytochemical studies, the inclusions did not stain with SBB or MPO, as would be expected with Auer rods. The small, irregular cytoplasmic granules stained with ACP and PAS, but the needle-shaped inclusions did not.

B5- and formalin-fixed, hematoxylin and eosin–stained sections and Wright-Giemsa–stained touch preparations of the diagnostic lymph node biopsy specimen were reviewed. There were no cytoplasmic inclusions.

Electron Microscopy

The cells had round to irregular nuclei with marginated heterochromatin and occasional small nucleoli. The cytoplasm contained numerous free ribosomes, rare segments of rough-surfaced endoplasmic reticulum, and small numbers of well-formed mitochondria. Some of the cells demonstrated membrane-bound elongate, cytoplasmic crystaline inclusions (Figs. 2 and 3). In particular, there was a linear substructure with a periodicity of 8 nm to some crystals (Fig. 3). In some areas, a fingerprint-like configuration was seen (Fig. 3, inset). Some inclusions appeared to be membrane bound, but others did not. Often, several crystals would appear within a single dilated cistern associated with electron-dense noncrystalline material.

Crystals in Lymphoma Cells
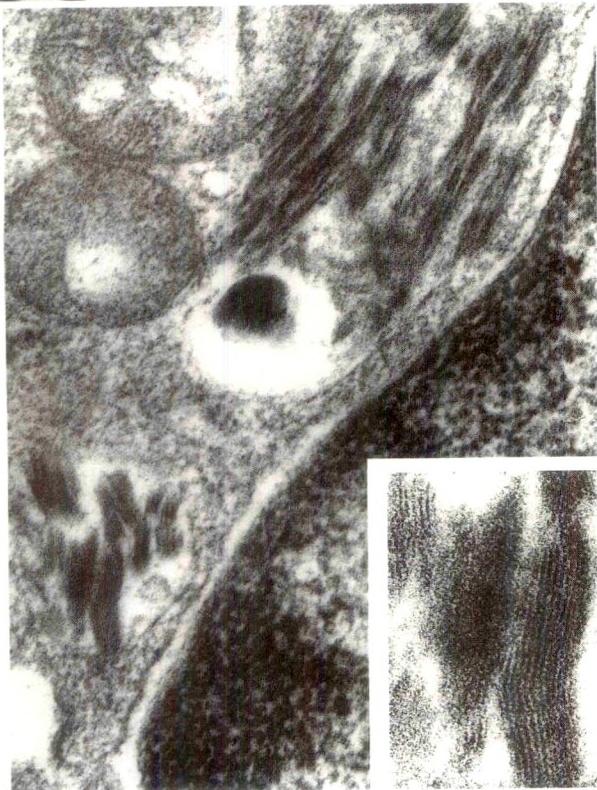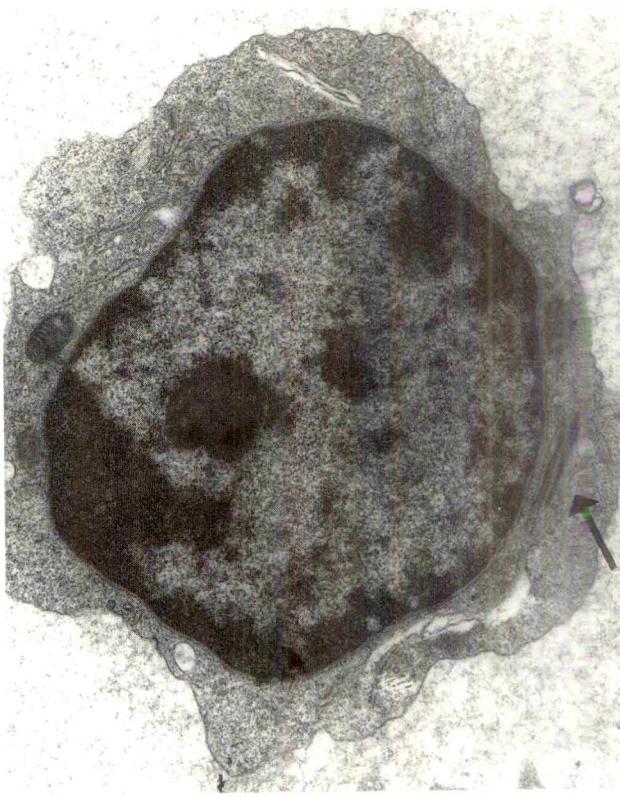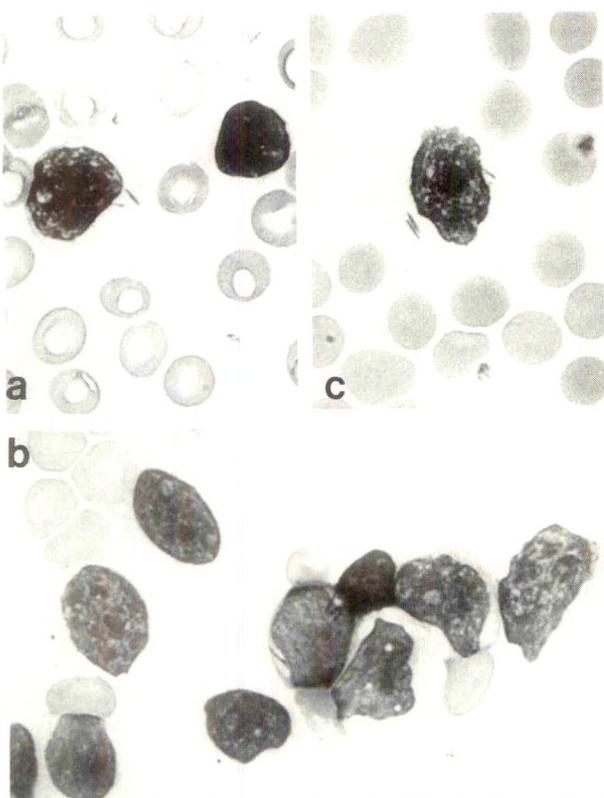
FIG. 1 (*upper left*). Wright-Giemsa–stained blood smear showing a lymphocyte containing needle-shaped cytoplasmic inclusions (*A* and *B*) and a disrupted cell with free-lying needle-like crystals (*C*). Original magnification (×1,000).

FIG. 2 (*upper right*). Electron micrograph of a lymphocyte demonstrating a paranuclear crystalline inclusion (*arrow*). Original magnification (×11,600).

FIG. 3 (*lower*). High-power electron micrograph of membrane-bound cytoplasmic inclusions showing linear substructure (×70,200). *Inset,* A "fingerprint" pattern with periodicity of 8 nm (×140,400).

Flow Cytometry

Flow cytometric analysis of peripheral blood lymphocytes showed a predominant B-cell population. Fifty-eight percent of the circulating lymphocytes were CD20 positive and expressed surface-bound kappa light chain (kappa–lambda ratio of 20:1). Thirty-seven percent of the circulating lymphocytes were T cells staining for CD2. The circulating lymphocytes did not stain significantly with CD5 (11%) or CD10 (4%). Flow cytometric analysis of bone marrow lymphocytes produced similar findings.

Immunocytology

Immunostaining demonstrated kappa, IgM, and CD22 positivity of the lymphoma cells, but there was no staining with antilambda, IgG, IgA, or CD3. The cytoplasmic inclusions did not stain with any of these reagents.

Immunoelectron Microscopy

Cytoplasmic kappa light chain staining was seen in most lymphoma cells; however, the results for lambda light chain were negative. The cytoplasmic crystalline structures showed no specific staining for kappa or lambda light chain.

DISCUSSION

Intracytoplasmic crystalline inclusions have been described and characterized in various lymphoproliferative disorders (Table 1). The intracytoplasmic needle-like crystals seen in the circulating lymphoma cells of our case did not stain with MPO, SBB, PAS, or ACP. Ultrastructurally, these crystalline structures were fibrillar, showed periodicity, and often were found within dilated cisterns. This is much like the description of the electron microscopic appearance of Auer rods seen in acute myeloid leukemia.[18,19]

The needle-like inclusions in our case differ from the large proteinaceous refractile crystals described in some cases of chronic lymphocytic leukemia, plasma cell tu-

mors, and one case of a B-cell lymphoma.[8-15] In addition to the obvious difference in light microscopic appearance and staining qualities, these cases ultrastructurally demonstrated rectangular or rhomboid electron-dense cytoplasmic inclusions that usually had squared or rounded edges, not splinter-like structures. Also, internal periodicity of these structures was noted only rarely. Several studies have shown specific immunoglobulin or light chain staining of these crystalline inclusions by light microscopic immunofluorescent or immunoelectron microscopic techniques.[8-10,14,15]

Intracytoplasmic structures that have a somewhat different light microscopic appearance but ultrastructurally resemble the crystalline inclusions seen in our case have been seen in the cytoplasm of blasts of acute lymphoblastic leukemia.[1-4] By light microscopic examination, these structures are multiple, round, and coarse and vary widely in staining characteristics with Romanofsky staining. They do not stain with PAS, MPO, SBB, or specific or nonspecific esterases; but, in at least two cases,[3,4] the inclusions stained for ACP activity. The authors reasoned that these structures probably represented abnormal lysosomes.

By light and electron microscopic examination, the crystalline inclusions in the current case appear to be remarkably similar to the Auer rods seen in acute myeloid leukemia. However, there are important histochemical and immunologic differences. Auer rods, which are fused lysosomal granules,[16,18] stain with MPO, SBB, ACP, and PAS, unlike the structures seen in our case. Of course, specific staining for immunoglobulin heavy or light chains is not seen in neoplastic myeloid cells or Auer rods.

Our case demonstrated light microscopic, ultrastructural, and immunologic findings almost identical to those described previously in four case reports of lymphoproliferative disorders. In 1974, Lagios and associates[20] described needle-like cytoplasmic inclusions in circulating neoplastic cells from an elderly man with chronic lymphocytic leukemia. There was no specific staining of inclusions for SBB, PAS, or MPO. The inclusions appeared ultrastructurally identical to those in our case. Similarly,

TABLE 1. COMPARATIVE MORPHOLOGY AND HISTOCHEMISTRY OF CRYSTALLINE INCLUSIONS SEEN IN LYMPHOPROLIFERATIVE DISORDERS

Subject Case	Auer Rods[16-19]	Crystals in CLL and Plasma Cell Tumors[8-15]	Auer Rod-Like Crystals in Other Lymphoproliferative Disease[20-22,29]
Azurophilic	Azurophilic	Azurophilic	Azurophilic
Splinter shaped	Splinter shaped	Rectangular	Splinter shaped
Negative MPO	Positive MPO	Negative MPO	Negative MPO
Negative PAS	Positive PAS	Positive PAS	Negative PAS
Negative ACP	Positive ACP	Negative ACP	1 of 2 cases positive ACP
Negative Ig immunostain	Negative Ig immunostain	Positive Ig immunostain	Negative Ig immunostain (1 case)

crystals were not demonstrated in the circulating neoplastic cells after chemotherapy was started.

Auer rod–like inclusions also have been reported in a case of prolymphocytic leukemia.[21] No staining of inclusions was demonstrated with PAS, SBB, MPO, nonspecific esterase, chloroacetate esterase, ACP, or beta-glucuronidase.

Two cases of Auer rod–like inclusions in multiple myeloma were described previously. In 1950, Steinman reported a case and demonstrated the absence of peroxidase activity.[22] In 1983, Raman and colleagues[29] described needle-shaped crystalline inclusions within malignant plasma cells of the bone marrow. There was no staining with PAS, SBB, MPO, or nonspecific esterase, but there was strong ACP and beta-glucuronidase positivity. Immunostaining showed strong IgA and kappa positivity in the cytoplasm of the tumor cells but no staining of the crystals.

To our knowledge, Auer rod–like intracytoplasmic inclusions have not been described previously as occurring in circulating neoplastic lymphoid cells of small cleaved follicular center cell lymphoma. Using immunoperoxidase light microscopic and immunoelectron microscopic techniques, we could not show that the inclusions were of an immunoglobulin nature. Ultrastructurally, these inclusions do not resemble the immunoglobulin crystals seen within the cells of some lymphoproliferative disorders.

Similar membrane-bound, needle-like crystalline structures have been described as occurring in other lymphoproliferative disorders.[20–22,29] These inclusions bear striking ultrastructural similarities to the Auer rods described in acute myeloid leukemia. Indeed, although no MPO or SBB activity has been demonstrated in any of these cases, a few studies have described some ACP and beta-glucuronidase activity, indicating that these structures probably are lysosomes. Although we could not demonstrate ACP or other lysosomal enzyme activity in the cytoplasmic inclusions in our case, their ultrastructural appearance strongly suggests a lysosomal origin.

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Clonal Immunoglobulin Gene Rearrangement in the Infarcted Lymph Node Syndrome

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The authors report a case of complete lymph node infarction in which a specific etiology could not be determined by morphologic or immunophenotypic studies; however, clonal rearrangement of the immunoglobulin gene was demonstrated by Southern blot hybridization of DNA extracted from the necrotic tissue. A subsequent lymph node biopsy later was diagnosed as malignant lymphoma, using morphologic, immunophenotypic and genotypic criteria. Identical clonally rearranged bands were present in DNA from both the infarcted nodal and the subsequent tissue biopsies. In the setting of lymph node necrosis, gene rearrangement studies may provide diagnostic information concerning clonality, even if morphologic and immunophenotypic studies are indeterminate for a lymphoproliferative process. (Key words: Lymph node infarction; Ischemic necrosis; Malignant lymphoma; Gene rearrangement) Am J Clin Pathol 1991;96:116–120

Spontaneous lymph node infarction is rare but when encountered it may present a significant diagnostic problem. The vasculature of lymph nodes provides a dual blood supply, with numerous anastomosing arterial branches.[1,2] Possible causes of infarction include vascular thrombosis, vasculitis, and mechanical vascular obstruction.[1–3] Rare cases of viral-like illnesses associated with lymph node infarction have been reported, but the mechanism involved in these cases is unclear.[4] The strong association of lymph node infarction with a subsequent diagnosis of malignant lymphoma recently has been stressed.[5–7] The diagnosis of malignancy in these situations often can be made only after a second biopsy. We report a case of complete lymph node infarction in which initial morphologic and immunophenotypic studies were nondiagnostic but in which clonal immunoglobulin gene rearrangement was demonstrated by Southern blotting in DNA extracted from the necrotic tissue. A subsequent biopsy was interpreted as malignant lymphoma by morphologic, immunophenotypic, and immunogenotypic

criteria, and this tissue contained the same rearrangement as that detected in the original biopsy. This study illustrates the potential utility of gene rearrangement studies in the diagnostic evaluation of infarcted lymph node specimens.

REPORT OF A CASE

A 77-year-old white man presented with a slowly enlarging mass in the left side of his neck. The lesion had been noticed for approximately three months but had grown more rapidly during the preceding week. The patient reported no fevers, night sweats, weight loss, or fatigue. The past medical history was remarkable for thyroid adenoma, myocardial infarction, stable angina pectoris, hypertension, transient cerebral ischemic attacks, and prostatic hyperplasia. On physical examination, a 3–4 cm, soft, mobile, slightly tender mass in the left side of the neck was palpated, as were several smaller (less than 1 cm) nodes. Laboratory data included evidence of a mild normochromic, normocytic anemia (hemoglobin 12.0 g/dL; hematocrit 36.6%). Results of remaining general screening laboratory studies were within normal limits, including the serum lactate dehydrogenase level (LDH 198 IU/L [normal 100–210 IU/L]). An incisional biopsy revealed complete infarction of lymph nodal tissue, as well as the surrounding pericapsular adipose tissue. Another biopsy was done one week later. The tissue from this biopsy was obtained from a different lymph node chain in the left side of the neck. Pathologic examination of the second specimen revealed malignant lymphoma.

MATERIALS AND METHODS

Biopsy specimens were fixed in buffered formalin, sectioned at 5 μm, and stained with hematoxylin and eosin for morphologic studies.

Immunophenotypic studies were performed on snap-frozen lymph node tissue by direct immunofluorescence microscopy. Reagents used included fluorescein-conjugated polyclonal anti-IgG, -IgM, -IgA, -IgD, -kappa, and -lambda (Kallestad, Inc., Austin, TX) and unconjugated monoclonal antibodies directed against the CD-20, -2, -3, -4, -8 determinants (B1, T11, T3, T4, T8, Coulter, Inc., Hialeah, FL); CD-24 and -5 determinants (BA-1, T101, Boehringer Mannheim, Indianapolis, IN); CD-22 and -7 determinants (Leu 14, Leu 9, Becton Dickinson, Mt. View, CA); CD-35 and -45 determinants (C3BR, leukocyte common antigen, DAKO, Santa Barbara, CA); and the CD-71 determinant (OKT9, Ortho, Raritan, NJ). Application of the monoclonal reagents was followed with application of a fluorescein-conjugated polyclonal anti-mouse G, A, M reagent (Coulter). Direct immunofluorescence microscopy was performed using a Leitz microscope (Rockleigh, NJ) equipped with a 450-W xenon lamp.

For immunogenotypic studies, DNA was extracted from tissues with phenol and chloroform, precipitated with ethanol, and dissolved. Five micrograms of DNA was digested with restriction enzymes (Boehringer Mannheim, West Germany). The *Bam*HI and *Hind*III restriction enzymes were used for immunoglobulin heavy chain gene (J_H) analysis, *Bam*HI was employed for kappa chain gene (CK) analysis, and *Bam*HI and *Eco*RI were used for T-cell receptor beta chain gene (TCR-B) analysis. Placental DNA served as a control. The digested DNA was electrophoresed on 0.8% agarose gel and transferred onto nitrocellulose paper by the method of Southern.[8] The membranes were hybridized with random-primed, P32-labeled probes, washed extensively, exposed to x-ray films for 3–72 hours, and developed. A 3.5-kilobase (kb) *Hind*III fragment TCR-B probe was supplied by Dr. Jeffrey Sklar (Stanford Medical School, Stanford, CA),[9] and 2.5-kb *Eco*RI fragment CK and 2.5-kb Sau3A fragment J_H probes were supplied by Dr. Philip Leder (Harvard Medical School, Boston, MA).[10,11]

RESULTS

Pathologic Findings

Histologic examination of the initial lymph node biopsy specimen showed massive ischemic necrosis of the lymph node and surrounding fibroadipose tissue. The capsule of the node was intact, with an adjacent thin rim of viable lymphoid tissue that was composed of small lymphocytes and sinus histiocytes. The majority of the node was represented by a confluent sheet of eosinophilic necrotic material with scattered pyknotic nuclei and areas showing the silhouettes of necrotic lymphocytes (Fig. 1). The

number of inflammatory cell infiltrates within the node was minimal; however, neutrophils were spread diffusely throughout the surrounding soft tissue. Granulomatous infiltrates, vascular thromboses, and vasculitis were not seen. A reticulin stain showed the residual collapsed reticulin skeleton.

The second lymph node biopsy showed focal effacement of nodal architecture by a follicular and diffuse proliferation of large, atypical lymphoid cells. In some areas the infiltrates were composed of a mixture of small cleaved and large cells; however, the majority of the neoplastic cells were large with cleaved nuclei, together with large noncleaved cells (Fig. 2). Although infarction of the second lymph node was not identified, the surrounding pericapsular fibroadipose tissue showed areas of ischemic necrosis.

The immunophenotypic studies of the initial biopsy specimen were difficult to interpret, presumably because of the extensive necrosis and poor antigenic preservation. The infarcted cells stained strongly with antikappa and antilambda light chain antibodies and expressed CD-22 (Leu-14); however, staining with other pan–B-cell markers (*i.e.,* CD-20 [B1], CD-24 [BA-1], and CD-35 [C3BR]) was uniformly negative. Several reagents, including anti-IgG, -IgM, and -IgA, showed nonspecific staining of the necrotic material, as well as the capsule and soft tissue. In addition, pan–T-cell markers were either uniformly negative or were uninterpretable because of nonspecific staining. The malignant lymphoma in the second specimen lacked expression of surface immunoglobulin but stained with CD-20, -22, -24, and -35, consistent with a B-cell lymphoma of follicular center cell origin.

Immunogenotypic studies were performed on both specimens as described. Identical clonally rearranged bands were detected with the J_H and CK probes in DNA extracted from both specimens, indicating a clonal B-cell proliferation (Figs. 3 and 4). The TCR-B blots revealed no clonal rearrangement in either study. These findings show that, despite the necrosis, there was preservation of a sufficient amount of DNA to permit successful immunogenotypic analysis.

DISCUSSION

The rarity of superficial lymph node infarction has been a topic of discussion in the literature for the last 50 years. In animal studies, it has been shown that even with occlusion of the lymph nodal vascular pedicle, perfusion to the capsule and subcapsular cortical tissues is maintained.[12,13] Initial reports of spontaneous lymph node infarction suggested vasculitis, vascular thrombosis, or mechanical vascular obstruction as possible etiologies.[1-3] Reported associations of infarction with viral-like illnesses accompanied with fever were described later.[4] In recent

Single Case Report
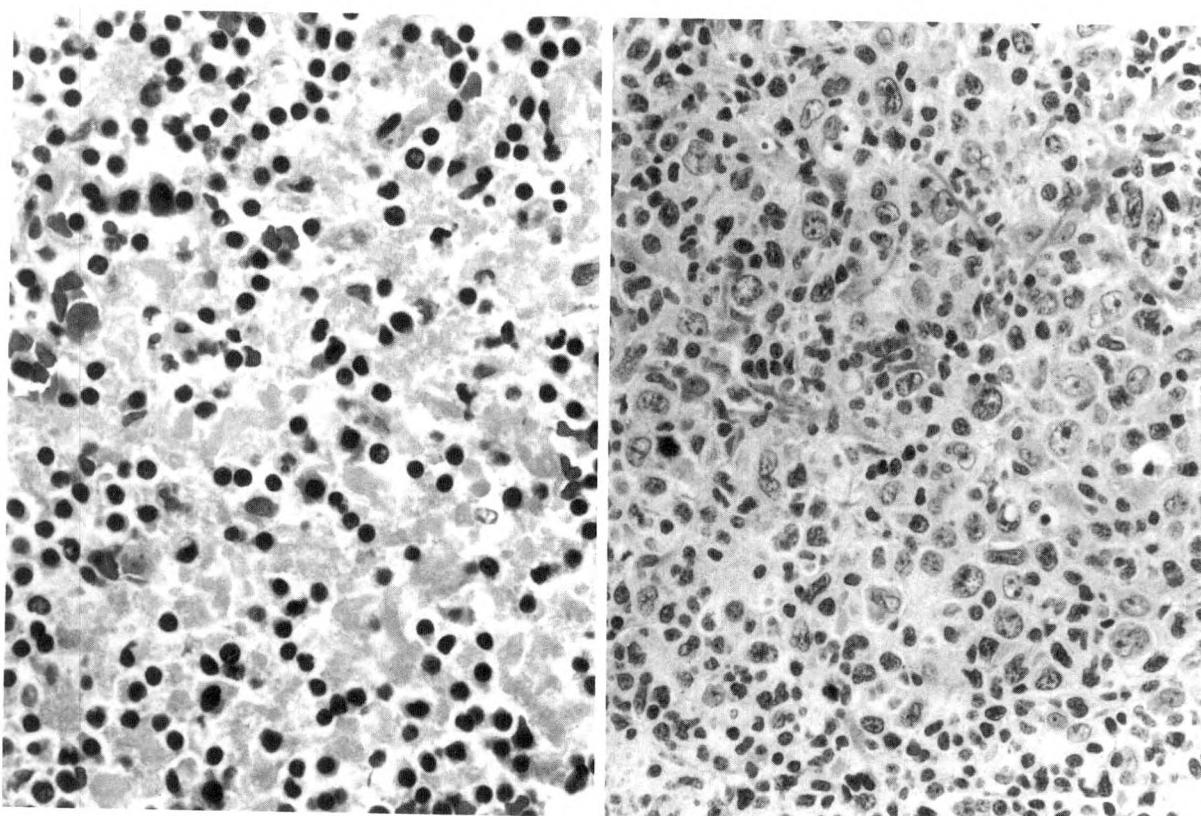
FIG. 1 (*left*). The center of the first lymph node biopsy specimen, containing confluent zones of coagulation necrosis and scattered pyknotic nuclei. Hematoxylin and eosin (×100).

FIG. 2 (*right*). The second biopsy specimen containing a neoplastic lymphoid proliferation with a follicular growth pattern comprised predominantly of large cleaved and noncleaved cells. Hematoxylin and eosin (×100).

years, the association of complete lymph node infarction as a herald of the development of malignant lymphoma has been stressed, although the cause for this phenomenon remains unknown.[5-7]

Gorelkin and Majmudar[14] reported a case of massive lymph node infarction in an elderly patient. After another biopsy two months after the initial biopsy, the patient was diagnosed with malignant lymphoma. Gorelkin and Majmudar thought that the initial biopsy was infarcted because of an unexplained benign process; they stressed the finding of residual reticulin framework in the infarcted node, as compared with the neoplastic node. They believed that reticulin staining in the necrotic tissue helped distinguish "benign" ischemic necrosis from tumor-associated infarction.

Cleary and colleagues[5] reported 16 cases of lymph node infarction in patients whose initial lymph node biopsy specimens showed complete ischemic necrosis. In this study, 13 of the 16 patients (80%) were diagnosed with malignant lymphoma after subsequent biopsies, which were obtained from two days to six months after the initial biopsy. One of the remaining three patients was later di-

agnosed with metastatic carcinoma after a second biopsy of an axillary lymph node. Apparently, no malignancy developed in the other two patients. The cause of infarction in these cases was not determined. The majority of the lymph nodes (70%) were from the head and neck region, and the most frequent histologic finding was that of a diffuse large cell lymphoma (6/13 cases; 46%).

Ballas and co-workers[15] studied 12 cases of "infarcted lymph node syndrome" and found that several clinical factors and laboratory data helped identify the cause of the infarction. In the Ballas series, all six patients with elevated serum LDH were diagnosed with lymphoma after subsequent biopsies that followed the initial biopsies by periods ranging from days to months. The study did not state how many of the six patients with normal LDH levels subsequently developed lymphoma. Additional factors in predicting a neoplastic outcome included advanced age, size of the nodes, duration of symptoms, and the presence and degree of anemia. In contrast, in the study by Cleary and associates, the serum LDH was reported to be normal in five cases in which such determinations were available, and all five were diagnosed with lymphoma after subse-

J_H

Bam H I   Hind III

C S1 S2   C S1 S2
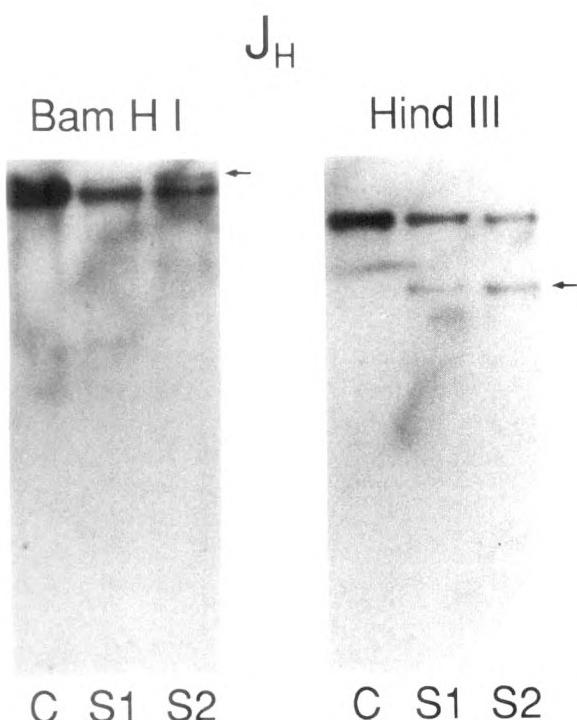
FIG. 3. Southern blot analysis of the immunoglobulin heavy chain (JH) gene using *Bam*HI and *Hind*III restriction endonuclease digestions. Germline bands are present in the control (*C*) and the specimen lanes (Si and S2). Identical clonal rearranged bands (*arrows*) are present on the *Bam*HI and *Hind*III digested lanes from the first (S1-infarcted) and second (S2-noninfarcted) specimens.

quent biopsies.[5] The serum LDH level in our patient was normal at the time of the initial biopsy. However, it appears that an elevation of the serum LDH in the setting of lymph node infarction may be a useful indicator to suggest lymphoma as a possible etiology. Nonetheless, a normal value certainly does not exclude that diagnosis.

In 1986, Maurer and colleagues[16] reported the results of a multicenter study of the infarcted lymph node syndrome. The analysis included a total of 51 cases in which infarction was present in lymph node biopsies. Lymphoma was found synchronously with the infarct in 14 cases. The remaining 37 cases, having a mean follow-up period of 48 months, showed the development of malignant lymphoma in a subsequent biopsy specimen in six cases (16%). The overall incidence of lymphoma in infarcted lymph nodes was approximately 40% (20/51 cases). The authors stressed the observation that the malignancies of all of these patients developed within two years of the initial biopsies. In these cases, the reticulin framework of the nodes was preserved, regardless of the etiology of the infarction. In this study, the lymphomas were more evenly distributed between small and large cell lymphoid neoplasms, compared with studies that previously reported a higher incidence of large cell lymphomas. These authors did not have laboratory data to support

the suggestions made by Ballas and colleagues[15] concerning the utility of the LDH level in determining the cause for the infarction.

Immunophenotypic data were not reported in early case reports and series on lymph node infarction. Recently, Norton and associates[17] studied 11 cases in which lymphoma was diagnosed at the time of or after lymph node infarction. These authors used monoclonal antibodies to "fixation-resistant leukocyte antigens" in paraffin-embedded tissue. In 8 of the 11 cases, the immunophenotypic studies provided evidence for a B- or T-cell proliferation, and these data paralleled results obtained with subsequent noninfarcted tissues. In the Norton study, immunophenotypic data suggested or supported a diagnosis of lymphoma, but clonality could not be determined immunologically. In our case, immunophenotypic studies were inconclusive. Although the majority of the residual viable and partially necrotic cells were CD-22-(Leu-14) positive, the remaining pan–B-cell markers were nonreactive, and the kappa and lambda stains were equally positive. In addition, several studies (heavy chain and T-cell markers) showed diffuse nonspecific staining of the capsule and surrounding soft tissues.

Immunoglobulin and T-cell receptor beta chain gene rearrangement studies have proven to be useful adjuvant

C_κ

Bam H I

C S1 S2
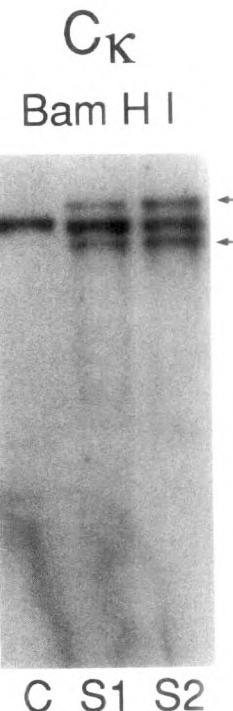
FIG. 4. Southern blot analysis of the immunoglobulin kappa light chain (CK) gene using *Bam*HI restriction endonuclease digestion. Germline bands are present in control (C) and specimen lanes (S1 and S2). Identical clonal rearranged bands (*arrows*) are present in both the first (S1-infarcted) and second (S2-noninfarcted) specimens.

procedures in the evaluation of lymphoid proliferations.[18] In this case, clonal immunoglobulin gene rearrangement was detected by Southern blot hybridization in the infarcted specimen. Moreover, the subsequent biopsy specimen, which was diagnosed as malignant lymphoma, contained identical clonally rearranged bands. The ability of DNA to withstand ischemic necrosis and prove suitable for Southern blot analysis is unknown. It is likely that gene rearrangement was detected because of the sensitivity of this technology in identifying a clonal population of cells. In theory, if one in 20 cells contained intact strands of DNA, gene rearrangement could be detected. Although antigen preservation may parallel DNA preservation, the interpretation of immunophenotypic studies may be limited by nonspecific staining and a lack of definite cell surface light chain restriction. This report illustrates another potential application of gene rearrangement studies in the evaluation of diagnostically difficult lymph node specimens and further emphasizes the need for obtaining fresh or snap-frozen tissue at the time of a lymph node biopsy.

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Original Article

Parvovirus B19 Infection of the Fetus
Histology and In Situ *Hybridization*

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Fetal tissues from 16 spontaneous abortions, two terminations, and one perinatal death, 18 of which were associated with maternal human parvovirus B19 infection, were examined for B19 infection by histology and *in situ* hybridization using a digoxigenin-labeled B19-DNA probe. In 15 spontaneous abortions and one termination, erythroblasts with intranuclear inclusions (lantern cells) reacted with B19-DNA by *in situ* hybridization. No internal or external fetal malformations were observed. Because 13 (86.7%) spontaneous abortions with lantern cells occurred between the 20th and 28th weeks of gestation, it is postulated that B19 infection may be a particular threat to the fetus during this stage of gestation. (Key words: Parvovirus B19; Abortion; Fetal infection; Histology; *In situ* hybridization) Am J Clin Pathol 1991;96:121–126

Human parvovirus B19, first identified by Cossart and colleagues,[1] induces aplastic crises in patients with sickle cell anemia and other chronic hemolytic anemias.[2] The most common clinical manifestation of B19 infection is erythema infectiosum, a disorder that can be accompanied by exanthema, arthralgia, polyarthritis, lymphadenopathy, and/or flu-like symptoms.[2–4] Vascular purpura is sometimes associated with acute B19 infection.[5] Recently, B19 has been shown to cause chronic bone marrow aplasia in immunodeficient patients.[6] Asymptomatic B19 infections apparently are common and represent as many as 80% of cases of known seroconversion.[3,4,7]

In pregnancy, B19 infection may harm the fetus by causing hydrops fetalis, a condition that leads to fetal loss in 5–20% of such cases.[3,4,7] Fetal complications following B19 infection have been reported at all stages of gestation,[7–17] and the time between maternal symptomatic B19 infection and onset of fetal complications can be as long as 12 weeks.[18] Erythroid precursor cells are the target cells of B19 infection.[19,20] In the fetus, viral infection of these cells can inhibit fetal erythropoiesis,[21] resulting in massive anemia and hydrops fetalis. B19 infection of the mother is diagnosed by detecting B19-specific IgM (anti-B19 IgM) in serum. In some cases, fetal B19 infection has been identified by detecting anti-B19 IgM or B19 antigen in cord blood[22] or B19 antigen in amniotic fluid.[15] Elevated alpha-fetoprotein also may indicate fetal B19 infection.[23]

B19-infected erythroblasts with intranuclear inclusions have been observed in fetal tissue in isolated cases.[11,21,22,24,25] In this report, we confirm the histologic finding of intranuclear inclusions in a greater number of fetuses that were aborted after maternal B19 infection. We also demonstrate B19-DNA in these cells by *in situ* hybridization using a digoxigenin (Dig)-labeled B19-DNA probe.

MATERIALS AND METHODS

Patients

We examined tissues of 17 fetuses (cases 1–17) aborted at various stages of gestation, and of one neonate (case 18) who died following birth trauma (Table 1). In all instances, acute maternal B19 infection was demonstrated before delivery. All 18 women (cases 1–18) were positive

TABLE 1. CLINICAL FINDINGS IN B19-ASSOCIATED FETAL DEATHS (CASES 1–16), B19-NEGATIVE FETUSES (CASES 17 AND 18) AND A NEGATIVE CONTROL (CASE 19)

Case	Maternal Age (years)	Week of Intrauterine Death	Autopsy Findings	Lantern Cells	B19-DNA Positive Tissues
1	26	25	Hydropic	Lungs	Lungs
2	33	24	Hydropic	Liver, spleen, heart, pancreas, muscle, skin	Liver, spleen, heart, pancreas, muscle, skin
3	21	23	Hydropic	Liver, spleen	Liver, spleen
4	24	24	Hydropic	Liver, spleen	Liver, spleen
5	30	26	Hydropic	Liver, bone marrow, skin	Liver, bone marrow, skin
6	34	33	Hydropic	Liver, spleen, bone marrow, heart, lungs, pancreas, kidneys	Liver, spleen, bone marrow, heart, lungs, pancreas, kidneys
7	41	25	Hydropic	Liver, bone marrow, kidneys, placenta	Liver, bone marrow, kidneys, placenta
8	25	20	Hydropic	Liver, spleen, bone marrow, pancreas, lungs, kidneys, placenta, skin, thyroidea, ovary	Liver, spleen, bone marrow, lungs, pancreas, kidneys, placenta, skin, thyroidea, ovary
9	42	21	Hydropic	Liver, spleen, bone marrow, pancreas, heart, kidney, lungs, thyroid gland, skin, muscle, ovary	Liver, spleen, bone marrow, pancreas, lungs, heart, kidneys, thyroid gland, skin, muscle, ovary
10	25	14	Anemic nonhydropic	Liver, spleen, bone marrow, heart, lungs, brain, placenta	Liver, spleen, bone marrow, heart, lungs, brain, placenta
11	35	24	Hydropic	Liver, spleen, heart, lungs, kidneys	Liver, spleen, heart, lungs, kidneys
12	18	24	Hydropic	Liver, lungs	Liver, lungs
13	26	28	Hydropic	Spleen, lungs, kidneys, muscle, kidneys, intestine	Spleen, lungs, muscle, kidneys, eye, intestine
14	37	23	Hydropic	Bone marrow	Bone marrow
15	27	18	Hydropic	Bone marrow	Bone marrow
16	29	12	Unknown*	Liver, bone marrow, lungs	Liver, bone marrow, lungs
17	33	17	Hydropic* polycystic kidneys	Liver, spleen, bone marrow, heart, lungs, kidneys	Liver, spleen, bone marrow, heart, lungs, kidneys
18	25	Perinatal death	Tentorial bleeding	Liver, spleen, bone marrow, heart, lungs, kidneys, brain	Liver, spleen, bone marrow, heart, lungs, kidneys, brain
19	29	15	Unknown†	Liver, spleen	Liver, spleen

* Pregnancy terminated.   † Cause of death unknown.

for anti-B19 IgM and IgG by ELISA.[26] In the final case (case 19), the mother tested negative for B19-specific antibodies. All patients were tested either because they had symptoms of erythema infectiosum, had contact with persons known to have B19 infection, or were admitted to the hospital because of fetal hydrops fetalis. The median age of the mothers was 29.5 years (range 18–42 years). Of the 18 fetal deaths, 1 (5.5%) occurred in the first trimester, 12 (66.7%) in the second, and 5 (27.8%) in the third.

The aborted fetus of a woman who tested negative for B19-specific antibodies (case 19) was used as a negative control.

Fetal Tissues

Tissues of the 17 fetuses, the neonate, and control (listed in Table 1) were obtained from obstetrics and gynecology hospitals and departments of pathology. Fetal tissues were either obtained fresh within 24 hours of delivery, fixed in buffered 4% formaldehyde, or embedded in paraffin.

Additional age-matched controls consisted of a panel of liver tissue from 33 aborted fetuses with unknown B19-antibody status that had been collected previously by the

Institute of Pathology, Munich, Germany, from cases of intrauterine death of unknown cause between the 18th and 36th weeks of gestation. All such tissues were examined for B19 infection by histology and *in situ* hybridization.

Histology

Two-micrometer slices of each tissue block were stained routinely with hematoxylin and eosin. For detailed localization of infected cells, serial sections were stained immunohistochemically using specific antibodies against collagen type IV (Eurodiagnotics, Leiden, Netherlands) and an indirect immunostaining procedure with the secondary antibody coupled with avidin-biotin complexes (Vector Laboratories, Inc., Burlingame, CA) to identify endothelial basement membranes.[27]

In Situ *Hybridization*

For *in situ* hybridization, slides with paraffin-embedded fetal tissues were deparaffinized in xylol and washed in 70% ethanol. The slides were incubated in 2 × SSC (stan-

dard saline citrate) (1×; SSC = 0.15 M NaCl; 0.015 M Na-citrate pH 7.5) at 70 °C, followed by 20 mmol TRIS-HCl pH 7.4, 2 mmol CaCl, and 2 μg/mL Proteinase K for 15 minutes at 37 °C. After incubation in phosphate-buffered saline (PBS) and 0.2% glycine for 10 minutes at room temperature, the slides were treated in PBS and 4% paraformaldehyde pH 7.0 for 10 minutes at room temperature, washed in H_2O, and incubated in increasing concentrations of ethanol (50–70–90%) for 1 minute each. Slides were heated (100 °C) in 0.1× SSC for 30 seconds and cooled in ice-cold 0.1× SSC. Finally, incubation in ethanols (50–70–90%) was repeated. For the hybridization solution (1 mL), we used 1 M TRIS-HCl pH 7.5 (9.9 μL), 0.04 M EDTA (24.8 μL), 5 M NaCl (118.8 μL), 0.5 μg/mL formamide (495 μL), 2% Ficoll® (9.9 μL; Pharmacia, Freiburg, Germany), 2% polyvinyl-pyrrolidone (9.9 μL; Sigma, St. Louis), 100 mg/mL bovine serum albumin (BSA) (9.9 μL), 5 mg/mL calf-thymus DNA (19.8 μL), 1 mg/mL Poly A (99 μL; Boehringer Mannheim, Mannheim, Germany), 10 mg/mL t-RNA (99 μL), and H_2O (104 μL).

For detecting B19-specific DNA in fetal tissue, we used a 700 base pairs (bp) PstI-fragment of the B19 genome that was attached to *Eco*RI and *Hind*III linkers (donated by H. Wolf, Munich, Germany) and cloned into plasmid Pgem₂PTM1. This plasmid was digested with *Eco*RI and *Hind*III and the B19 insert separated and purified using standard methods. The B19 probe was labeled with digoxigenin (Dig) based on random primed incorporation of Dig-labeled desoxyuridin-triphosphate as described by the manufacturer. As a negative control, a DNA clone of another parvovirus, rheumatoid arthritis virus (RA-1) (supplied by D. VanLeeuwen, Abbott Laboratories, North Chicago, IL) was used.

For hybridization, 10 ng/μL of Dig-labeled B19 DNA and RA-1 DNA probe were added to 10 μL/slide of the hybridization solution, heated at 100 °C for 4 minutes and cooled in ice-cold water. Finally, this mixture was added to the slide, covered with a silicon-covered cover glass and incubated for 24 hours at 37 °C. The cover glass was removed, and washed. The immunological staining reaction, based on alkaline phosphate-conjugated polyclonal sheep anti-Dig Fab fragments, was performed as suggested by the manufacturer (Boehringer Mannheim). The slide was mounted in Kaisers Glycerine-gelatine (Merck, Darmstadt, Germany) and examined under a light microscope (Zeiss, Oberkochen, Germany).

RESULTS

Acute B19 infection was confirmed serologically in 18 pregnant women (cases 1–18). The group included 15 cases of spontaneous abortions, 2 terminations (following

pathological ultrasound findings in 1 case and on maternal request in the other), and 1 perinatal death. Erythroblasts with intranuclear inclusions were identified in 16 (88.9%) fetuses. Because these erythroblasts reacted positively by *in situ* hybridization with B19 DNA, the inclusions were regarded to be specific for B19 infection. These infected erythroblasts resemble a Chinese lantern; hence, we refer to them as "lantern cells."

Clinical Findings

The median age of the 16 women with B19-associated fetal loss (cases 1–16) was 29.2 years (range 18–42 years). In 4 cases, symptoms typical of acute B19 infection were observed. Erythema infectiosum was reported in 2 (12.5%) cases, in the 13th (case 10) and the 18th (case 3) weeks of gestation. Two (12.5%) other women, during the 18th (case 14) and the 11th (case 16) weeks of gestation, reported contact with persons known to have erythema infectiosum, whereas the remaining 12 women (75.0%) were asymptomatic until the onset of fetal complication.

One pregnancy was terminated at the mother's request during the 12th week of pregnancy (case 16); ultrasound findings were unavailable. In the remaining 15 cases of spontaneous abortion (cases 1–15), intrauterine death occurred between the 14th and 33rd weeks of gestation; 13 fetuses (86.7%) died between weeks 20 and 28.

Gross Autopsy Findings

Hydrops fetalis was present at autopsy in 14 of the 15 (93.3%) spontaneous abortions. The hydrops was generalized, although each fetus was affected to a different extent. One fetus (case 10) was nonhydropic but was severely anemic; interestingly, massive hydrops fetalis had been diagnosed by ultrasound examination two weeks before intrauterine death. However, at autopsy the hydrops had disappeared, probably because therapeutic intrauterine transfusion had been performed.

External malformations were present in only one fetus (cheiloschisis; case 5). None of the other cases had either external or internal malformations. No evidence of fetal B19 infection was found in case 17, a pregnancy that was terminated because of polycystic kidney disease in the 17th week of gestation, or in case 18, a neonate who died of birth trauma (intracerebral bleeding). Ultrasound monitoring of the pregnancy was normal in the latter case.

Histologic Findings

In 16 fetuses, B19 infection was detected by the identification of erythroblasts with intranuclear alterations (lantern cells). The nuclei of these erythroblasts were eosinophilic, enlarged, and ballooned; hematoxylin and

eosin staining showed marginal inclusions. In fetal tissues of the 15 cases of abortion and the one terminated pregnancy (Table 1, cases 1–16), lantern cells were demonstrated by routine histologic staining. Such lantern cells were detected in various fetal tissues (Table 1) but were found mostly in small capillaries. In the fetal liver, spleen, and bone marrow (Fig. 1), lantern cells were found in the stroma. In two fetuses, lantern cells were detected intravascularly in the placenta. The number of lantern cells identified by light microscopy varied among the different cases. The use of immunohistochemistry with specific antibodies against collagen type IV for the localization of lantern cells in tissues other than liver, spleen, or bone marrow revealed an exclusive localization of these cells within vascular lumens (Fig. 2). In the nonhydropic fetus (case 10), only a few lantern cells were detected. Tissues from the neonate (case 18), the fetus used as a negative control (case 19), and the fetus from the pregnancy that was terminated because of polycystic kidneys (case 17), were negative for lantern cells.

In liver tissues of 33 fetuses aborted between the 18th and 36th weeks of gestation with unknown B19-antibody status, no lantern cells were detected.

In Situ *Hybridization*

B19-DNA was demonstrated by *in situ* hybridization using a Dig-labeled 700 bp PstI fragment of the B19 genome in tissues from all 16 fetuses with histologically identified lantern cells (Fig. 3). No hybridization signals
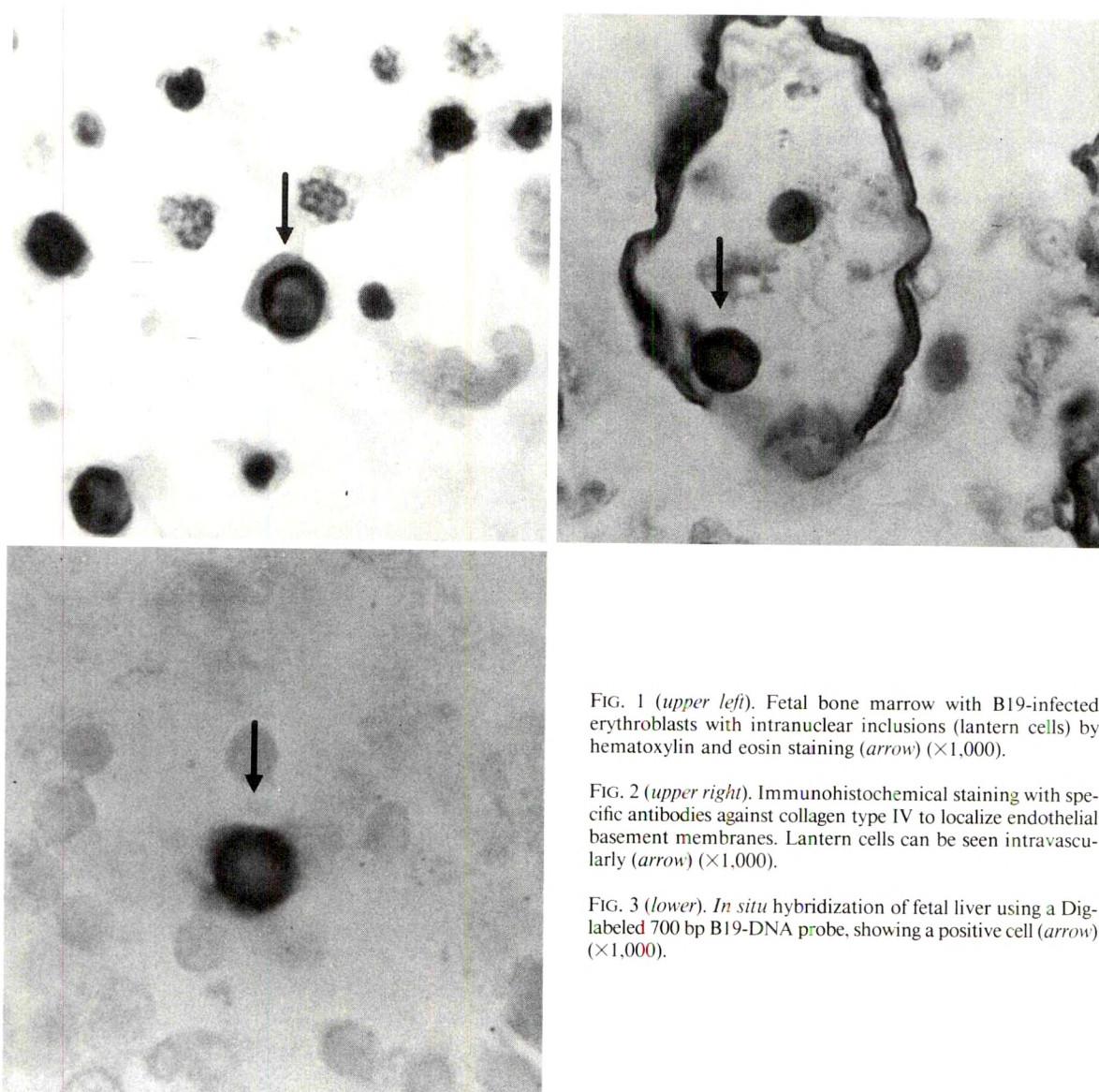
FIG. 1 (*upper left*). Fetal bone marrow with B19-infected erythroblasts with intranuclear inclusions (lantern cells) by hematoxylin and eosin staining (*arrow*) (×1,000).

FIG. 2 (*upper right*). Immunohistochemical staining with specific antibodies against collagen type IV to localize endothelial basement membranes. Lantern cells can be seen intravascularly (*arrow*) (×1,000).

FIG. 3 (*lower*). *In situ* hybridization of fetal liver using a Dig-labeled 700 bp B19-DNA probe, showing a positive cell (*arrow*) (×1,000).

were obtained in B19-positive tissues (case 6) using the Dig-labeled RA-1 DNA probe. No other cell type was shown to contain B19-DNA. The number of positive cells detected by *in situ* hybridization was slightly higher than that obtained by histologic routine staining. In autolytically altered tissue areas, positive B19-DNA signals were seen in cells even when a typical lantern configuration was no longer recognizable. Tissues from the neonate (case 18), the fetus used as a negative control (case 19), and the fetus from the pregnancy terminated because of polycystic kidney disease (case 17) were negative for B19 DNA.

To test specificity of the assay, liver tissues of 33 aborted fetuses with unknown B19-antibody status were tested for B19-DNA by *in situ* hybridization. None of the cells from these tissues were positive for B19-DNA.

DISCUSSION

Like some other viral infections (rubella virus, varicella zoster virus, cytomegalovirus) in pregnancy, B19 infection endangers the fetus. The risk of fetal loss in women with acute B19 infection is estimated to be between 5% and 20%,[4,7] with most abortions occurring between the 10th and the 20th weeks of pregnancy.[4] In contrast, in our study most B19-associated fetal deaths occurred between the 20th and the 28th weeks; the vulnerability of the fetus to B19 virus during this period can be explained by the short fetal red cell survival and the rapidly expanding red cell volume that occurs at that time.

Malformations following fetal B19 infection have been reported in only one case in early pregnancy[16]; accordingly, we observed no malformations or abnormalities in B19-infected fetuses. One case of cheiloschisis was seen, but as this abnormality frequently occurs spontaneously, it cannot be linked to B19 infection. In one study, B19-DNA was detected in fetal myocardial cells by *in situ* hybridization, indicating infection of the myocardium,[22] but we found no indication of myocardial infection in myocardial tissues of five fetuses with known B19 infection. No cells other than erythropoietic cells were positive for B19-DNA by *in situ* hybridization in any of the tissues examined in this study.

In one fetus from the mother with polycystic kidney disease (case 17) and in the neonate who died of intracerebral bleeding (case 18), lantern cells were not detected histologically, and *in situ* hybridization was negative, despite the fact that acute maternal B19 infection was confirmed serologically. In these two cases, B19 virus seems not to have been transmitted transplacentally; the fetal outcome could not be related to the maternal infection.

Immunohistochemical staining of endothelial basement membranes revealed that infected cells were intravascular except in liver, spleen, and bone marrow. The target cell

of B19 infection apparently is the erythroblast, and the organs with the greatest number of infected erythroblasts (lantern cells) were the liver, spleen, and bone marrow.

In situ hybridization techniques are used widely for confirming the presence of viral DNA. Hazards, expense, and relative instability of radioactive labels associated with these techniques have led to a search for alternative, nonradioactive, means of DNA labeling, such as biotin and acetylaminofluorene for B19-DNA *in situ* hybridization.[28] Dig-labeled B19-DNA probes have been used for dot blot hybridization and have been shown to be highly sensitive and specific for detecting as little as 0.1 pg B19 DNA.[29] In addition, Dig-labeled B19-DNA probes are stable for several months. The convenience offered by such probes allows further routine use in the examination of paraffin-embedded fetal tissues.

In view of the apparently high risk to the fetus, all women with an acute illness that resembles a B19 infection or women who have been in contact with B19-infected patients should be tested serologically for anti-B19 IgM. If confirmed positive, monitoring of the pregnancy is recommended.[4] In the event of intrauterine death, fetal tissues should be examined histologically for lantern cells. If such cells are present, fetal B19 infection should be confirmed by *in situ* hybridization.

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Original Article

Early Use of Indirect Immunofluorescence for the Detection of Respiratory Syncytial Virus in HEp-2 Cell Culture

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Respiratory syncytial virus is detected in cell culture by the presence of cytopathic effect. To detect RSV before cytopathic effect is usually seen, slides were evaluated retrospectively from 482 HEp-2 cell cultures on days 2–4 after inoculation. Indirect immunofluorescent staining detected RSV in 57 of 94 cultures that eventually were found positive by cytopathic effect. In an additional 19 cases that ultimately showed no cytopathic effect, RSV also was detected. In 15 of the latter cases, the presence of RSV was confirmed in the original specimen. Use of indirect immunofluorescence can be used to augment the sensitivity of cell culture for the detection of RSV because cytopathic effect may not always be evident. (Key words: Respiratory syncytial virus; Immunofluorescence; HEp-2 cell culture; Antigen detection; Cytopathic effect) Am J Clin Pathol 1991;96:127–129

Respiratory syncytial virus (RSV) infection is the most common cause of lower respiratory tract infection in infants. It is diagnosed by detection of antigen in nasopharyngeal secretions or by viral isolation from these secretions using cell culture. Detection of RSV antigen by immunofluorescence in exfoliated nasal epithelium[1–3] or by enzyme immune assay of antigens in nasopharyngeal secretions using commercially available kits[4–7] is useful in the care of potentially infected patients because results may be available within hours. In contrast, the detection of RSV in cell culture may require as long as two weeks. In addition, the low cost of antigen detection by immunofluorescent or immunoenzyme methods is an advantage over the relative expense of cell culture. However, some investigators question the specificity and sensitivity of antigen detection by these means. In some settings, viral isolation may be required to subtype RSV isolates[8] or to test the isolates for sensitivity to antiviral compounds.[9] Cell culture, the universal standard, also is needed as a basis for comparison in the evaluation of antigen detection techniques. Thus, there is still a need for cell culture in RSV diagnosis. However, it may be possible to improve the sensitivity of current detection methods by using antigen detection, in conjunction with cell culture, to identify RSV independent of its cytopathic effect.

RSV usually is detected in cell culture by the presence of a characteristic cytopathic effect (CPE), but RSV antigens are detectable in cell culture before CPE develops.[10–12] In addition, the presence of other viruses or biochemical changes in the medium may obscure the CPE of RSV. Immunofluorescent staining of cell culture can detect the growth of RSV in the absence of CPE.

To evaluate the sensitivity of immunofluorescent staining of cell culture for the detection of RSV at a time when CPE was not likely to be present, HEp-2 cells were examined by immunofluorescence on days 2–4 after inoculation with clinical specimens. As expected, the use of immunofluorescence early in the culture sequence (early immunofluorescence) reliably detected virus that subsequently developed typical CPE. More importantly, early immunofluorescence detected RSV in cases where CPE was never observed. Enzyme immunoassay of the original specimen was used to confirm most of these results as true positives.

MATERIALS AND METHODS

Over a two-year period (1984–1986), 482 nasopharyngeal aspirates were inoculated into Rhesus monkey kidney, human embryonic kidney, WI-38, and HEp-2 cell culture for the isolation of viruses using standard techniques and

commercially available cell culture materials.[4] Specimens were kept in test for 2 weeks. A single slide was made from one of the two tubes of HEp-2 cells by scraping the cells from the tube with a pipette. Slides were made on days 2–4 after inoculation. Only one slide was made from each culture. These slides were stored at −70 °C and later stained using an indirect technique with bovine anti-RSV (Wellcome Reagents Limited, Beckenham, England) and fluorescence conjugated antibovine antibody (Kirkegaard and Perry Laboratories, Gaithersburg, MD). All isolates of RSV detected by CPE in HEp-2 cells were confirmed by the same indirect immunofluorescent technique. Slides for RSV immunofluorescence were made only on days 2–4 and when RSV CPE was detected. No slides were made at the end of the two-week inoculation period. Specimens from this time period also were stored at −70 °C and later were examined by RSV-selective enzyme immunoassay (EIA) available from either Kallestad,[6] Ortho,[4] or Abbott laboratories[5] as part of an ongoing evaluation of these assays. The early immunofluorescence slides were stained and read several months later, independent of the final isolation results or the results of the EIAs.

RESULTS

The sensitivity of early immunofluorescent staining for the detection of RSV relative to culture was 61% (Table 1). An important observation was the detection of RSV by early immunofluorescence on slides obtained from 19 negative cultures. The original specimens associated with 15 of these 19 slides had positive EIAs. All 15 positive EIAs had either a positive blocking test,[4–6] a second positive EIA by a kit from a second manufacturer, or a positive direct immunofluorescent test on the original specimen, if no blocking test was done. Three specimens were subjected only to the Ortho EIA test and were negative, whereas one specimen was not run in any EIA test. Seven of these 15 confirmed specimens had other viruses isolated in cell culture: two were enteroviruses, three were adenoviruses, and two were influenza B virus.

TABLE 1. EARLY IMMUNOFLUORESCENCE *VERSUS* CULTURE IN 482 NASOPHARYNGEAL ASPIRATES

Culture	Early Fluorescence	
	+	−
+	57	37
−	19*	369

* Fifteen of nineteen cultures negative confirmed by enzyme immunoassay or direct fluorescence of nasopharyngeal aspirate.

DISCUSSION

The diagnosis of RSV by the detection of CPE in cell culture can be difficult. The development of RSV CPE is dependent on glutamine[13] and magnesium concentration.[14] Thus, if the biochemical conditions of the medium are not sufficient, syncytium formation may not take place. In addition, HEp-2 cells are heteroploid and can grow past a confluent monolayer, making the detection of CPE difficult. Furthermore, other viruses (such as Adenovirus or some of the enteroviruses that grow in HEp-2 cells) can obscure the development of RSV CPE.[2,4,5,15] RSV occasionally may not be apparent in cell culture because of a lack of quality culture in commercially available cells for their ability to develop syncytia. Because of these problems with cell culture and because of the speed of antigen detection systems such as immunofluorescence or EIA, such methods of antigen detection may be preferred to culture for the diagnosis of RSV infections.

Interestingly, in this study, four positive early immunofluorescent studies were not confirmed by culture or EIA. These could represent false-positive results, the presence of nonviable virus in exfoliated nasal epithelium that was inoculated into the HEp-2 cells, or a failure of the less-sensitive Ortho enzyme immunoassay.[4,16] If the early fluorescence results had been known at the time, the cultures could have been reexamined by immunofluorescence or a more-sensitive EIA, before the cultures were discarded, to confirm the presence of RSV.

Others have found CPE to be less reliable than immunologic antigen detection methods. In a study of 40 frozen specimens that were positive for RSV on initial inoculation, 35 were positive on reinoculation of spun cell cultures stained for RSV using an immunoperoxidase method on day two after inoculation.[17] Of these 35 positives, only 26 developed CPE on standard cell culture. An additional four cases were detected using immunoperoxidase staining of standard cell culture material on day 18. The finding of additional positive results using an antigen detection method in the absence of CPE is consistent with our study.

Given our observations and the information presented here, a strong argument can be made that either early immunofluorescence of HEp-2 cells or immunofluorescence of all HEp-2 cells that fail to show CPE should be performed before the cells are discarded. In addition, other cell lines available in a virology laboratory that may not show typical CPE could be used to isolate RSV using the same techniques. Previous studies of WI-38 and Rhesus monkey kidney cells for the culture of RSV[11] have revealed results equal to those obtained with HEp-2. Other investigators have found Rhesus monkey kidney cells to be superior to HEp-2 cells, whereas a combination of these

cell lines has proven to be superior to either line used alone.[18]

In most laboratories, failure to detect RSV often is due to the absence of CPE. Based on this experience and frequent comments in the literature about viral overgrowth, we propose that routine immunofluorescence of cell culture material be used in all future studies of RSV. Further, given the speed of antigen detection using patient specimens, we advocate the use of antigen detection (fluorescence or EIA) methods with nasopharyngeal material as an initial procedure, combined with the application of a similar technique at the termination of the culture sequence.

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Catalase-Negative *Listeria monocytogenes* Causing Meningitis in an Adult

Clinical and Laboratory Features

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A 63-year-old previously healthy woman presented with acute meningitis. Cultures of the cerebrospinal fluid yielded a serotype 1/2a isolate of *Listeria monocytogenes* that was biochemically typical in all respects, other than the reproducible lack of catalase production. During therapy, the patient developed oculomotor dysfunction that was attributed to an abscess in the internal capsule. This case report documents the existence of catalase-negative *L. monocytogenes* indicating that catalase production should not be a strict criterion for identification of Listeria. Furthermore, this clinical experience extends *in vitro* and experimental animal studies indicating that catalase production is not a necessary virulence factor for invasion by Listeria. (Key words: Catalase; *Listeria monocytogenes;* Meningitis; Virulence) Am J Clin Pathol 1991;96:130–133

Listeria monocytogenes, a facultative intracellular pathogen, is a recognized cause of invasive infection. Catalase production is one of the characteristics typically relied upon for separating Listeria from other morphologically similar bacteria.[1–3] Catalase, along with superoxide dismutase, has been implicated in the pathogenicity of *L. monocytogenes.*[4] We describe a case of listeriosis in which the isolate, a catalase-negative strain, expressed marked pathogenicity in an immunocompetent patient, producing both meningitis and abscess formation.

REPORT OF A CASE

In July 1989, a 63-year-old woman developed an illness characterized by fever, chills, headache, and generalized weakness. After three days of self-medication with a nonsteroidal anti-inflammatory agent, she sought

the care of her physician, who presumptively diagnosed a urinary tract infection and prescribed a course of cephalexin. When the patient's symptoms persisted, she sought care again the next day and was admitted to a hospital.

Her temperature on admission was 39.4 °C, and physical examination revealed no localizing findings. Except for an elevated white blood cell (WBC) count, routine laboratory studies were unremarkable. She was begun on a regimen of cephapirin 2 g IV every 6 hours. The next day the patient became lethargic and confused, developed nausea and vomiting, and was noted to be dysarthric and to have hemiparesis of the right side. She continued to be febrile on the third hospital day, received gentamicin, and was transferred to the Oklahoma Medical Center.

On arrival at our institution, the patient was restless and intermittently confused. She complained of headache. Her fever remained at 39.4 °C, and nuchal rigidity was present. Her WBC was 24.5×10^9/L (24,500 cells/μL) with an increase in immature neutrophils. Chest x-ray was normal. Computer assisted tomographic scan of the head with and without contrast enhancement demonstrated no mass effect or hemorrhage. Lumbar puncture obtained cerebrospinal fluid that contained RBC 75 $\times 10^6$/L; WBC $3,850 \times 10^6$/L (72% PMN); glucose 1.9 mmol/L (34 mg/dL) (blood glucose was 9.9 mmol/L [179 mg/dL]), and protein 2.54 g/L (245 mg/dL); Gram's stain was negative. Blood cultures (two sets employing lysis/centrifugation and biphasic media) were obtained. Supportive therapy and a regimen of parenteral penicillin and chloramphenicol were begun.

The next day, the patient was alert and oriented, complaining of a stiff neck and photophobia. Growth of a catalase-negative Gram-positive rod from CSF was reported. Antimicrobial chemotherapy was changed to ampicillin 3 g IV every 4 hours and gentamicin 120 mg IV every 8 hours. The microorganism isolated from CSF was subsequently identified as a catalase-negative *L. monocytogenes.*

Clinical improvement continued until the sixth hospital day, when

the patient was found to have photophobia, limitation of upward and lateral gaze, and right upper extremity weakness. Suspicion that this may have been due to aminoglycoside-induced neuromuscular blockade led to discontinuation of gentamicin therapy. Lumbar puncture was repeated, and all parameters were improved; Gram's stain was negative, and culture remained negative. Magnetic resonance imaging (MRI) (Fig. 1A) of the head with gadolinium contrast enhancement demonstrated ventriculitis and areas of probable brain abscess in the left optic radiation and internal capsule. Antimicrobial therapy was changed to a regimen of ampicillin/sulbactam 3 g IV every 4 hours.

The patient was discharged after 32 days of parenteral antimicrobial therapy with continuing therapy with oral amoxicillin with clavulanic acid. Resolution of the abscesses was documented by a repeat MRI (Fig. 1B) approximately 10 weeks after the initial study. A spinal tap showed normalization of all CSF parameters. She received an additional month of amoxicillin/clavulanic acid therapy. Other than improving lower extremity weakness, no sequela from the infection have been seen on close follow-up through nine months after presentation.

DISCUSSION

Infection with *L. monocytogenes* in adults most commonly manifests as meningitis.[5,6] Sudden onset is usual, unlike the subacute onset in our patient. Nuchal rigidity and fever are common. Tremors, seizures, and coma also may occur. CSF glucose levels are low in 40% of cases, blood cultures are positive in 60–70%, but CSF Gram's stains generally are negative.[5]

Frequently attacking the very young and aged, *L. monocytogenes* causes sporadic disease, especially during summer months. The portal of entry is thought to be the gastrointestinal tract. Epidemics have been linked to refrigerated food. Focal infections consisting of osteomyelitis, endophthalmitis, pulmonary infection, septic arthritis, and hepatic abscess rarely occur in immunocompromised hosts. The preferred treatment is a regimen of ampicillin. The addition of gentamicin therapy may result in synergistic activity because of increased bacterial uptake of the aminoglycoside.[5] We are unaware of data to support the use of beta-lactam/beta-lactamase inhibitor combinations in listeriosis.

L. monocytogenes is described as a small, Gram-positive, pleomorphic rod that is motile at 25 °C, beta-hemolytic, catalase-positive, and is a facultative anaerobe.[2,3,7] The isolate from this case fulfilled these criteria, except for catalase production, and also possessed the biochemical characteristics shown in Table 1. The identification of the organism was confirmed by the Centers for Disease Control (Atlanta, GA), where it was serotyped as 1/2a. Additional characterization in our laboratory included cellular fatty acid analysis, which was consistent with *L. monocytogenes* (data not shown).

The unusual finding in this case is the lack of catalase production. The catalase test has been described as a way to differentiate *L. monocytogenes* from diphtheroids,

TABLE 1. CHARACTERISTICS OF THE PATIENT'S ISOLATE AND THREE SPECIES OF LISTERIA

	Patient's Isolate	*L. monocytogenes*	*L. innocua*	*L. ivanovii*
B-hemolysis	+	+	0	+
Camp test	+	+	0	0
Acid production from:				
L-arabinose	0	0	0	0
L-rhamnose	+	+	0	0
Lactose	+	0*	+	+
Mannitol	0	0	0	0
Sorbitol	0	0	0	0
Sucrose	0	0	0	0
Voges-Proskauer	+	+	+	+
Hippurate	+	+	+	+

* See reference 3 for data on Listeria species.

Streptococcus species and enterococci. In *Bergey's Manual of Systemic Bacteriology,*[3] Seeliger and Jones recommend this test to differentiate Listeria from streptococci and Lactobacilli. They note that catalase production may be absent because of nutrient deficiency in the media and that negative strains rarely occur naturally. We are aware of a case of neonatal sepsis due to catalase-negative Listeria (also confirmed by the CDC) occurring recently (personal communication, Joseph M. Campos, Children's Hospital National Medical Center, Washington, DC). The frequency of encountering catalase-negative clinical isolates may be underestimated because of under-recognition. In CSF smears, Listeria can be mistaken as corynebacteria, streptococci, and even *Haemophilus influenzae.*[2] Bortolussi and colleagues,[2] suggest the use of catalase activity, along with Gram-stain morphology and motility, to separate Listeria species from Enterococcus and group B streptococci.

Noting the many similarities between *L. monocytogenes* and *Streptococcus agalactiae,* Kontnick and colleagues[8] reviewed the laboratory tests used to distinguish these organisms. Twenty-six commonly used tests were used to compare ten beta-hemolytic strains of each organism. While stating that absence of catalase essentially rules out *L. monocytogenes,* they note that both catalase and motility have been negative on subcultured organisms. Other authoritative references typically use a combination of characteristics to separate Listeria from seven other genera.[7] However, catalase production generally is a prominent feature, and lack of catalase activity causes divergence in these schemes from Listeria.[1,7] A compilation of tests and the usual reactions seen are presented in Table 2.

There is not universal agreement that catalase, along with superoxide dismutase, contributes to the pathogenicity of this organism. Production of these enzymes may explain why it is able to escape the respiratory burst in
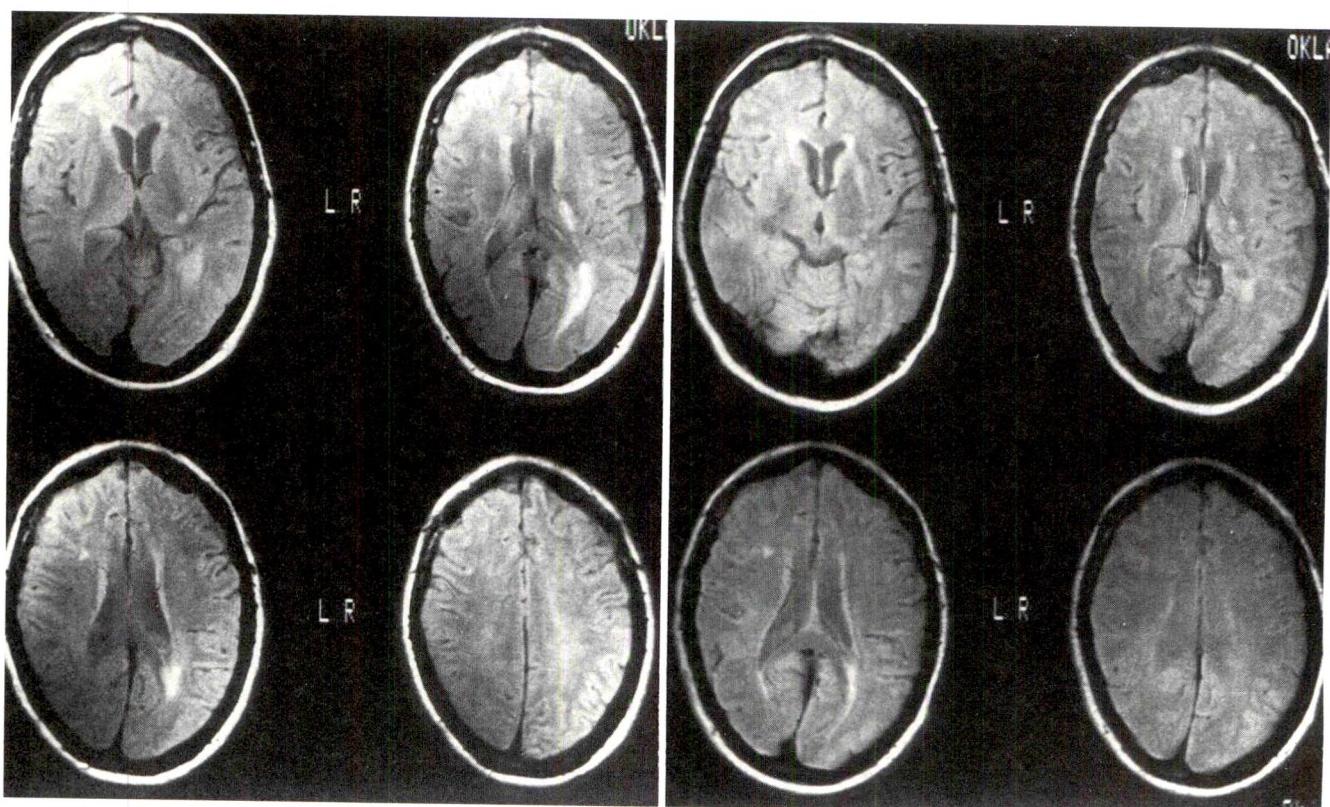
FIG. 1 (*left*). Magnetic resonance imaging (MRI) obtained after patient exhibited focal neurological symptoms. Present are abscess in both the left optic radiation and the internal capsule. (*right*). Follow-up MRI showing resolution of abscesses. The patient was free of the limitation of upward and lateral gaze and the right upper extremity weakness that had prompted the initial MRI.

monocytes and proliferate there. Investigations supporting a role for catalase in virulence of Listeria include that of Bortolussi and colleagues,[4] who found *L. monocytogenes* had both higher catalase activity and intracellular survival rates in the log phase than in the stationary phase of growth. Evasion of the phagocyte's oxidative products was attributed to the production of both enzymes. Welch and co-workers[9,10] found decreased virulence in strains with

no catalase activity and either decreased or increased superoxide dismutase activity. The catalase-positive strains survived in a medium containing superoxide radicals, whereas the negative variant, despite high levels of superoxide dismutase, was killed. In contrast, LeBlond-Francillard and associates[11] developed a transposon-induced catalase-negative mutant of *L. monocytogenes* and compared it with the catalase-positive parent strain. No difference in either the LD_{50} of mice or the recovery of Listeria from liver and spleen was seen with the mutant compared with the parent strain. Despite the controversy as to whether or not catalase production in *L. monocytogenes* contributes to its virulence in experimental systems, a catalase-negative strain produced meningitis and cerebral abscesses in our patient. The virulence of this organism is readily apparent in this case, and awareness, especially among laboratorians, of catalase-negative Listeria is important for proper diagnosis and therapy.

TABLE 2. USUAL DIFFERENTIATING CHARACTERISTICS BETWEEN *L. MONOCYTOGENES* AND OTHER GRAM POSITIVE ORGANISMS

	L. monocytogenes	*Group B Streptococci*	*Coryne Bacterium*	*Enterococci*
Gram Stain	*Pleomorphic Rod*	*Cocci*	*Pleomorphic Rod*	*Cocci*
Catalase	+	0	+	0
Motility	+	0	0	0
B hemolysis	+	0	+	+/0
Palisading	+	0	+	0
Hippurate hydrolysis	+	+	0	0
6.5% NaCl tolerance	+	0	0	+

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Neural Network Analysis of Serial Cardiac Enzyme Data

A Clinical Application of Artificial Machine Intelligence

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There has been a recent resurgence of interest in the study and application of computerized neural networks within the broad field of artificial intelligence. These "intelligent machines" are modeled after biological nervous systems and are fundamentally different from the many computerized expert systems that previously have been introduced as clinical decision-making aids. The authors describe a neural network designed and trained to predict the probability of acute myocardial infarction (AMI) based on the analysis of paired sets of cardiac enzymes. The neural network predicted 24 of 24 (100%) AMIs and 27 of 29 (93%) No-AMIs when compared with a pathologist's interpretation of the patient's laboratory data ($P < 0.000001$). The authors attempted to validate the network's diagnoses by two independent methods. When compared with echocardiogram and EKG for diagnosis of AMI, the neural network agreed with the cardiologist's interpretation in 12 of 14 (86%) AMIs and 1 of 3 (33%) No-AMIs, but the correlation was not statistically significant. Using autopsy outcome for validation, the neural network agreed with the anatomic evidence in 24 of 26 (92%) AMIs and 4 of 6 (67%) No-AMIs ($P = 0.001$). The authors conclude that neural networks can be successfully applied to the analysis of cardiac enzyme data and suggest that broader applications exist within the domain of clinical decision support. (Key words: Neural network; Artificial intelligence; Computers; Computer-assisted diagnosis; Myocardial infarction) Am J Clin Pathol 1991;96:134–141

Intelligent decision making involves the application of previous knowledge to the analysis of new data to arrive at a reasonable solution to a problem. The field of artificial intelligence attempts to implement this complex process using a nonbiological substrate, such as a computer. Intelligent machines of this sort may be useful in circumstances where an on-site expert is not immediately available, such as in remote locations or in critical situations where a decision must be made and acted upon immediately. In clinical medicine, artificial intelligence has not enjoyed widespread application, despite the prevalence of such critical situations and the often limited previous knowledge base on which those in attendance must act. We describe here an artificial intelligence program that has been trained to recognize acute myocardial infarction (AMI) by serial cardiac enzyme values.

Various applications of artificial intelligence to clinical decision support have been reported.[1–4] Realizing that the diagnosis of AMI frequently is a matter of pattern recognition, we have applied neural network technology to the task, adopting a fundamentally different approach than traditionally has been used.[5]

The majority of artificial intelligence programs available in clinical medicine make use of an algorithmic process for decision making.[6–8] In the simplest approach, an expert clinician's knowledge on a particular problem is distilled to a hierarchy of facts and rules. A knowledge engineer designs an inference engine that selectively invokes the rules necessary to distinguish between likely interpretations of the facts known to exist in a particular clinical situation. Rule-based expert systems of this sort are difficult to create and maintain and have a tendency to be rather inflexible in their approach to a problem. They often require the user to provide answers that are not available before a particular node in the decision tree can

be traversed during the course of an analysis. As a refinement on this method, some programs make use of a core knowledge base of pathophysiologic causal relationships that are used to supplement an algorithm that matches the patient's clinical information to a second knowledge base of disease profiles. These programs must be capable of resolving competing hypotheses that would explain a patient's condition, and often are confounded by variations in the clinical presentation of disease or the presence of multiple diseases.[9]

Neural networks are intelligent machines that are fundamentally different from rule-based or causally related expert systems. They are hardware or software emulations of biological nervous systems, formed by many interconnected artificial neurons. The artificial neurons are modeled after biological neurons, receiving excitatory or inhibitory synaptic input from a previous layer of neurons, summing the resulting signals, and firing output to the next layer of neurons (Fig. 1). Because the neural synapses are algebraically weighted, each neuron, in effect, performs a discriminant function analysis on its input. Because the output of the neuron will vary according to its level of excitation, it may or may not contribute to the input on the next layer of neurons. This "on/off" neuronal dichotomy provides that different discriminant functions may be applied to different problems and results in tremendous flexibility in problem solving at a level that is not easily achieved in purely rule-based systems. This also characteristically allows the neural network to perform well in tasks of pattern recognition but only at the expense of mathematical exactness. (In a financial institution, for example, a trained neural network would be well suited to evaluate the likelihood that an applicant would default on a loan but would do poorly at calculating an amortization schedule for repayment of that same loan).
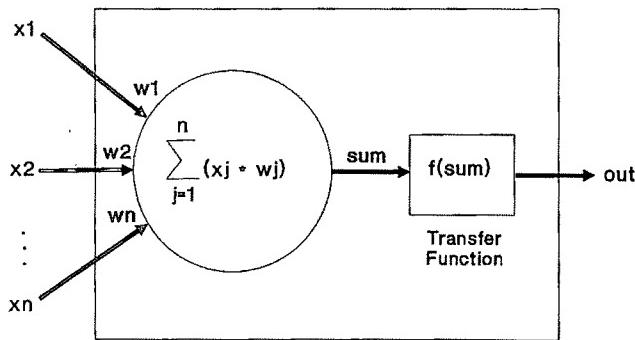

FIG. 1. Schematic representation of the artificial neuron. Afferent vectors (x) representing the output from a previous layer of neurons are algebraically weighted (w) and summed. A mathematical transfer function (f[sum]) is applied to the sum to determine the strength and/or frequency of neuron efferent firing (out).

Other unusual attributes of neural networks include their ability to perform successfully in situations in which some of the data (or even some of the neurons) may be missing. In these situations, which generally would be fatal to rule-based expert systems, massive parallelism imparts a high degree of fault tolerance and allows the neural network to arrive at reasonable solutions via alternate neural pathways.[10] The functional similarities to a biological nervous system often are striking.

Like biological systems, but unlike rule-based expert systems, the neural network must first be trained to perform well. On designing the network, each value of input data generally is assigned to a different neuron on the "input layer." One or more "hidden layers" of neurons are created, along with an "output layer." The output layer may consist of a single neuron, most useful for producing a single yes/no or graduated probability response,[11] or it may consist of many such neurons and be capable of much better resolution in its analyses. Upon creation, neurons from one layer generally are synapsed to neurons of the subsequent layer. The synapses often are assigned random excitatory or inhibitory algebraic weighting factors. Because of this, in its initial configuration, the network possesses no machine intelligence. The network is educated by presenting it with an extensive "training set" of data containing both input values and expected solutions for each type of problem that the network is being trained to analyze. The resulting signals on the output layer are compared with the expected values in the training set, and the weighting factors on each synapse are adjusted accordingly to minimize the difference between the neural network's calculated solution and the expected solution. By multiple iterations through the training set, synaptic weighting factors eventually are refined, such that the network arrives within a defined tolerance limit of the expected solution for each problem in the set. In so doing, certain neural connections become noncontributory to the solution, whereas others are strongly reinforced. Ultimately the "intelligence" of the network resides within the matrix of synaptic weighting factors. In practice, the trained network uses this matrix of previous experience to calculate solutions to new problems that are similar to those it has seen before.

Recognizing that this methodology may have broad application in clinical medicine, we undertook an evaluation of neural network technology in an area of some practical importance. Our specific objective was not to develop a computerized diagnostician, but rather to determine if neural network methods, when applied to the analysis of serial cardiac enzymes for the diagnosis of acute myocardial infarction, could correlate favorably with expert human analysis.

Original Article

TABLE 1. EXPERT'S CRITERIA FOR DIAGNOSIS OF AMI

A. Serum cardiac enzymes
   1. A serial rise and/or fall in CK and
   2. At least 1 sample with CK-MB $\geq$ 4% and CK-BB $\ll$ CK-MB
  or
   1. Significantly elevated LDH with serial rise and
   2. At least 1 sample with LDH-1 > LDH-2.
B. Echocardiogram/electrocardiogram
   1. ECHO showing definite focal wall motion abnormality
  or
   2. EKG showing definite Q waves meeting the Minnesota Criteria
     with or without associated ST-T wave changes

MATERIALS AND METHODS

The study was performed retrospectively in three phases. Phase 1 included neural network design and development of an appropriate training set. The data chosen for analysis included two identical serum cardiac enzyme (CE) panels, together with a time interval representing the elapsed hours between the two panels. The CE panel included creatine phosphokinase (CK), CK-MM (%), CK-MB (%), CK-BB (%), lactate dehydrogenase (LDH), LDH-1 (%), LDH-2 (%), LDH-3 (%), LDH-4 (%) and LDH-5 (%). All isoenzyme determinations were performed by agarose gel electrophoresis with densitometry. Over a ten-week period, 875 consecutive CE panels were reviewed to choose representative cases for the training set. Of these, 152 panels representing serial determinations from 47 patients were selected as diagnostic by virtue of serial CE alone, including 30 patients with AMI and 17 patients without AMI (No-AMI). Criteria for CE diagnosis are listed in Table 1. Paired permutations of the serial values

for each patient were then generated to expand the training set. Patients having three CE panels, for instance, would produce three paired CE sets (Panel-1:Panel-2, Panel-1: Panel-3, Panel-2:Panel-3), each with a different time interval between sets. Of the 239 paired CE sets thus generated, we limited analysis to those with a time interval between the two panels of 48 hours or less because this subset showed a sufficiently dynamic range of changing patterns that was not enhanced over longer time intervals. The final training set contained 185 such paired CE sets.

The network emulator chosen was Brainmaker 1.7 (California Scientific Software, Sierra Madre, CA). The network design included 21 input neurons representing the paired CE set data and the time interval in hours. This propagated to 11 neurons in a single hidden layer. Finally, a single neuron formed the output layer, producing a normalized value (0–1) interpreted as the probability of AMI (Fig. 2). To prevent any single neuron from too rapidly becoming saturated above its excitational threshold, and to impart a wider dynamic range to the system, each neuron was given a sigmoidal rather than step-wise or linear transfer function.

The matrix of synaptic weighting factors for the training set was calculated using a back-propagation, supervised learning algorithm. Training occurred under MS-DOS 3.2 on an IBM compatible 80386-based desk-top microcomputer with 2 Mbyte of RAM. More than 20,000 iterations through the entire training set were required to achieve a 10% error tolerance. With a 16 MHz microprocessor clock speed, the network training was completed within approximately four hours.

Tot CK-1
% CKMM-1
% CKMB-1
% CKBB-1
Tot LDH-1
% LDH1-1
% LDH2-1
% LDH3-1
% LDH4-1
% LDH5-1
Tot CK-2
% CKMM-2
% CKMB-2
% CKBB-2
Tot LDH-2
% LDH1-2
% LDH2-2
% LDH3-2
% LDH4-2
% LDH5-2
Delta Hours

AMI Probability
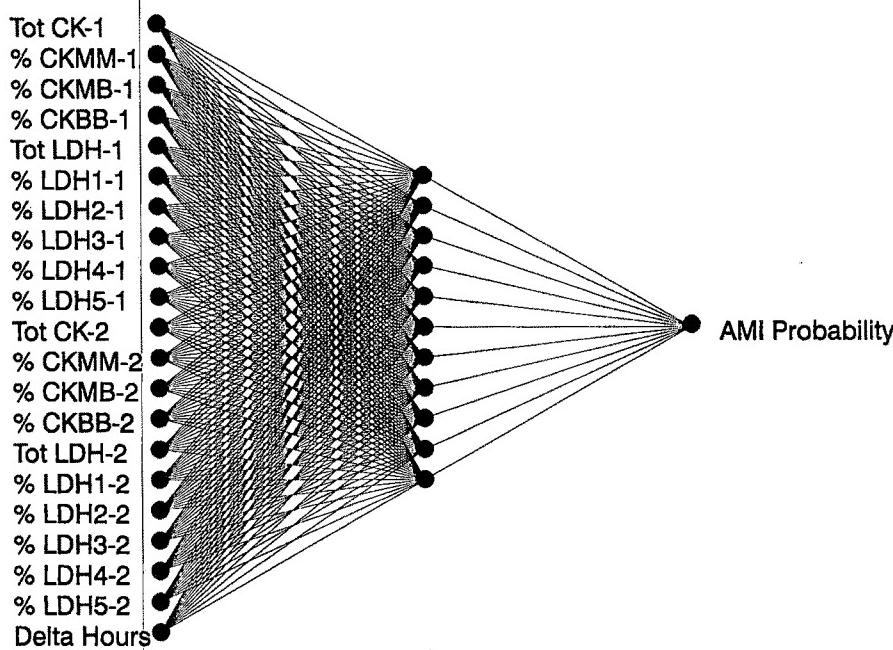
FIG. 2. Neural network architecture. Twenty-one neurons representing serial cardiac enzyme values form the input layer (*left*). Each input neuron is synapsed to 11 neurons, forming a single hidden layer (*center*). Each hidden layer neuron, in turn, stimulates a single neuron in the output layer (*right*), producing a normalized value interpreted as the probability of acute myocardial infarction.

In Phase II of the study, we tested the trained neural network by having it analyze 53 new paired CE sets, abstracted from consecutive CE determinations collected over a three-week interval. These data were collected retrospectively in the same manner as the training set and represented serial CE panels from ten patients with AMI and eight patients with No-AMI. The patients chosen for testing were excluded from the training set to guarantee that the network was tested naively. Unlike the process of supervised learning used to educate the network, testing involved only a single pass of each paired CE set through the trained network and was quite fast. For that reason, testing was performed on a 6 MHz IBM AT microcomputer with 512 Kbyte RAM under IBM-DOS 3.2, and required less than 1 second to analyze all 53 data sets in a batch mode. Because the network was trained to a 10% error tolerance, output probabilities of less than 0.1 were interpreted as No-AMI, probabilities of 0.9 or greater were interpreted as AMI, and all intermediate values were interpreted as indeterminate by network analysis. The network's interpretation for each paired CE set was then compared to the pathologist's interpretation for all CE panels from the same patient.

In Phase III of the study, we attempted to validate the trained neural network's analysis using independent methods of diagnosing AMI. Because the network was trained only to analyze cardiac enzymes, it was necessary to see if the network were truly successful at diagnosing AMI, or if it were successful only at recognizing the CE patterns that it had been taught were associated with AMI. To this end, we evaluated echocardiogram and EKG (ECHO/EKG) data, as well as autopsy material.

Retrospective serial CE determinations were available on nine patients who underwent one or more ECHO/EKG studies within two days of the first CE panel. The ECHO/EKG data were reviewed in a blinded fashion by a cardiologist (JH) with no knowledge of clinical history, CE values, or the neural networks analysis. Prior echocardiograms and electrocardiograms were evaluated where available. If serial studies were unavailable, ECHO/EKG changes that represented old myocardial infarction could not be distinguished from acute infarction and were, therefore, attributed to acute infarction. The criteria used for ECHO/EKG diagnosis of AMI are listed in Table 1. For each case, the cardiologist ranked the probability of AMI from 0 to 1. Because the resulting wide range of probabilities included more indeterminate values (by network training standards: $0.1 \leq$ output < 0.9) than diagnostic values, the data were transformed, assigning a probability of 0.5 or greater to the AMI category and less than 0.5 to the No-AMI category. This resulted in a diagnosis of AMI in seven patients and No-AMI in the remaining two. A total of 17 paired CE sets were available for neural network analysis from these 9 patients. The network's assessment on each of these was compared to the cardiologist's ECHO/EKG diagnosis on the same patient.

The second and final validation step was a retrospective review of autopsy material. Three hundred fifty-one consecutive autopsy records were reviewed to find 150 cases in which the postmortem examination of the heart included a sufficiently detailed gross description and enough microscopic sections to definitely diagnose or exclude AMI. The last hospital admission records were reviewed for these cases, and CE data were abstracted. Of these, 22 patients were found having two or more complete CE panels obtained within a 48-hour time interval near death and who, therefore, were eligible for neural network analysis. A total of 32 paired CE sets were available from these patients. Hematoxylin and eosin-stained glass slides were reviewed from each case, and additional hematoxylin-basic fuchsin-picric acid (HBFP)–stained slides were prepared on cases that showed no myofibrillar necrosis, interstitial hemorrhage, or leukocytic infiltration to exclude early myocardial ischemic damage.[12] The age of an infarction was estimated by the criteria of Mallory and colleagues.[13] Based on this and the dates of CE determinations and death, the diagnosis of AMI was assigned in cases in which the age of the most recent component of an infarction was estimated to be within about one week of the second CE panel. Cases in which there was no infarction or the age of infarction was estimated to be greater than about 1–2 weeks were included in the No-AMI diagnosis category. Using these criteria, 17 patients were assigned to the AMI category and 5 to the No-AMI category. These anatomic diagnoses were compared to the neural network's diagnosis for each paired CE set.

Nonparametric statistical tests of correlation were used to compare the neural network's diagnosis for each paired CE set with the expert's diagnoses. Because the network's output (a continuous variable between 0 and 1) was being compared to a dichotomous variable (0 or 1) representing the expert's interpretation of the CE or autopsy data, a point-biserial correlation test was used. For the ECHO/EKG data, for which paired continuous variables were compared, the Spearman rank correlation test was used. The level of significance was 0.01.

RESULTS

Results are summarized in Table 2. The trained neural network diagnosed AMI in 24/24 (100%) paired CE sets that were positive for AMI by expert CE criteria and No-AMI in 27/29 (93%) sets that were negative for AMI by

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**TABLE 2. SUMMARY OF NEURAL NETWORK'S
DIAGNOSES, COMPARED WITH PATHOLOGIST'S
INTERPRETATION OF CARDIAC ENZYME DATA (CE),
CARDIOLOGIST'S INTERPRETATION OF
ECHOCARDIOGRAM AND EKG DATA,
AND REVIEW OF AUTOPSY SLIDES**

Expert's Data Set	No. of Patients/ No. of CE Sets	Correct Diagnoses	Indeterminate Diagnoses	Incorrect Diagnoses
CE	18/53	51	2	0
ECHO/EKG	9/17	13	1	3
Autopsy	22/32	28	3	1

the same criteria. Only 2 of the 53 total paired CE sets produced an indeterminate probability ($0.1 \leq$ output < 0.9), and both of these were from No-AMI cases. The network's diagnoses correlated significantly with the expert's CE interpretations ($P < 0.000001$, Fig. 3). The distribution of the network's output probabilities for each group was narrow. For the expert AMI group, the mean and standard deviation were 0.998 and 0.003, respectively. When excluding the two sets that produced indeterminate probabilities from the expert No-AMI group, the mean and standard deviation were 0.021 and 0.023, respectively.

The network diagnosed AMI in 12/14 (86%) paired CE sets from patients who met ECHO/EKG criteria for AMI and diagnosed No-AMI in 1/3 (33%) sets from patients judged negative for AMI by ECHO/EKG criteria. One paired CE set from a patient judged negative by ECHO/ EKG was misdiagnosed as AMI by the network, with a probability of 0.999. This patient showed a mild elevation of total CK (286—319 U/L) with persistently elevated CKMB (13% − 12%) and no LDH isoenzyme "flip." The network diagnosed two paired CE sets from a single ECHO/EKG AMI patient as No-AMI, assigning a probability of AMI at 0.005 and 0.002. Although the CE data
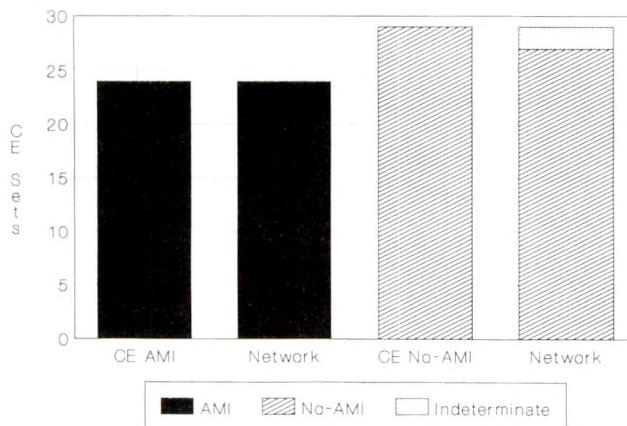
were not suggestive of AMI, the patient had a past history of multiple myocardial infarctions with dilated cardiomyopathy and chronic atrial fibrillation, rendering the diagnostic value of echocardiogram for AMI low. One additional paired CE set from an ECHO/EKG No-AMI patient produced an indeterminate probability. The network's diagnoses did not significantly correlate with the expert's ECHO/EKG interpretation ($P = 0.736$, Fig. 4).

When evaluating data from the autopsy cases, the network correctly diagnosed AMI in 24/26 (92%) paired CE sets from patients with anatomic evidence of AMI. The network correctly diagnosed No-AMI in 4/6 (67%) paired CE sets from patients lacking anatomic evidence of AMI. Three paired CE sets gave indeterminate results, including two patients with AMI and one patient with No-AMI. The network misdiagnosed AMI in the only paired CE set from one patient, assigning a probability of AMI at 0.990. Interestingly, this patient died from complications of idiopathic systemic amyloidosis. At autopsy, her heart showed perivascular amyloid deposits, with severe patchy fibrosis and ischemic changes, but no acute myocardial necrosis, hemorrhage, or leukocytic infiltration (Fig. 5). The network's diagnoses correlated significantly with the autopsy findings ($P = 0.001$, Fig. 6).

DISCUSSION

We have demonstrated the successful application of artificial machine intelligence, using a back-propagation neural network, to a common problem in clinical medicine. In so doing, several important observations emerged. First, and most importantly, the neural network's solutions to a clinical problem agreed with the solutions of the human expert to a strikingly high degree. At first glance, that may not seem significant because the network's interpretation of CEs was compared to the inter-


FIG. 3. Comparison of neural network analysis of paired serum cardiac enzymes (Network) with pathologist's interpretation of all cardiac enzymes from the same patients (CE).
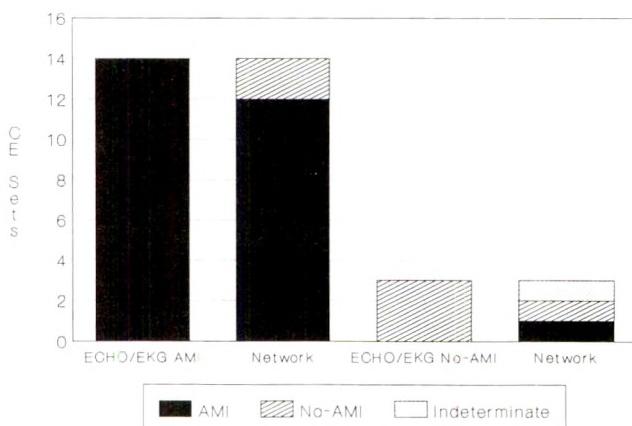

FIG. 4. Comparison of neural network analysis of paired serum cardiac enzymes (Network) with cardiologist's interpretation of echocardiographic and EKG data from the same patients (ECHO/EKG).
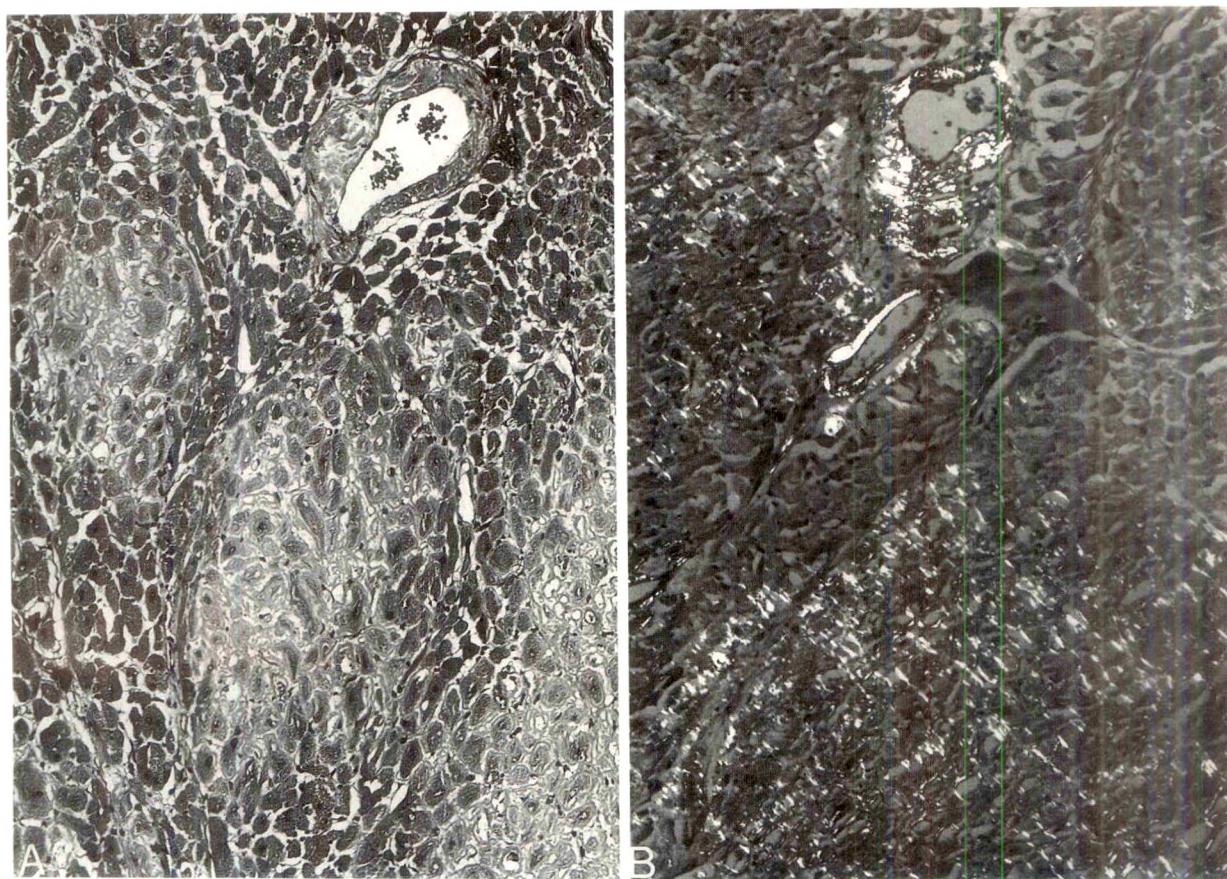
FIG. 5. Histology of myocardium in a patient who died of systemic amyloidosis, representing the single false-positive diagnosis made by the neural network among 32 autopsy cases studied. *A.* Hematoxylin and eosin section shows patchy myocardial ischemic damage without evidence of acute infarction. *B.* Polarization microscopy of congo red stained section confirms the presence of amyloid protein surrounding blood vessels and individual myocardial fibers. Original magnification ×100.

pretation of the same expert who designed the network. It is important to remember, however, that the expert did not program a series of interpretive rules into the network.
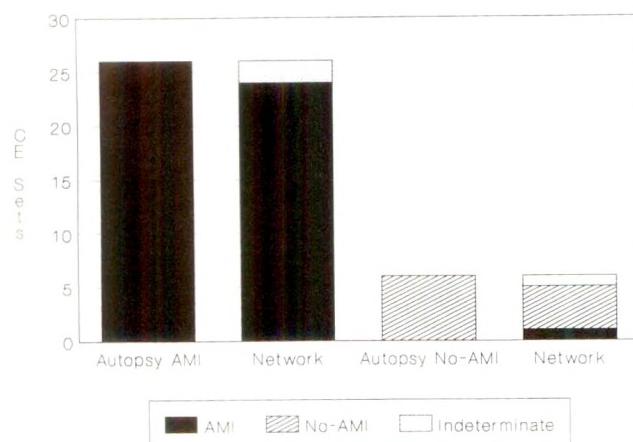
FIG. 6. Comparison of neural network analysis of paired serum cardiac enzymes (Network) with histologic interpretation of the heart in the same patients at subsequent autopsy (Autopsy).

The network programmed itself by adjusting weighting factors until its output matched the expert most closely. The fact that the expert and the network agreed so frequently on the test set data is a testament to the success of the neural network model and says nothing about the diagnostic accuracy of either the network or the expert.

The second important observation is that the neural network was generally quite "confident" of its solutions, arriving at probabilities that were well within the 10% tolerance limits required to complete training. This is evident from the distribution of output probabilities, averaging 0.998 for the AMI group and 0.021 for the No-AMI group. This is partly due to the nature of the cases chosen for training. The training set deliberately consisted mostly of fairly straightforward examples and avoided cases in which the expert interpreting the cardiac enzymes could not be certain of the diagnosis. It did, however, contain examples that could "fool" casual analysts, including massive skeletal muscle injury, cerebral infarction, congestive heart failure, and pulmonary infarction. However, some examples of the same sort were included in

Original Article

the test set, and the network was able to distinguish AMI from these other situations.

It is noteworthy that only 4% of paired CE sets in the test group produced indeterminate results, and that there were no misdiagnoses in the group. Analysis of the indeterminate cases highlights the network's "thought process" (Table 3). In one paired CE set, the total CK remained less than 50 U/L for both samples drawn 24 hours apart, but the LDH rose from 1,430 to 1,576 U/L, with LDH1 = LDH2 = 29% for the second sample. While this did not meet the expert's criteria for AMI, the network assigned a 0.887 probability of AMI to the case. The other indeterminate result involved a persistently low total CK with 100% MM, but a slight rise in total LDH from a baseline elevation of 607 to 842 U/L, along with a rise in LDH1 from 25% to 29%. The network calculated the probability of AMI at 0.110 for this case.

In addition to correlating with the expert's interpretation of the CE data and showing an overall confidence in its analyses, the neural network produced diagnoses that generally were correct as judged by two different methods of independent validation. The choice of ECHO/EKG as a means of validation was dictated by the retrospective nature of the project because this combination of tests was available most frequently for the patients studied. However, it is interesting to note that the network pro-

duced a much tighter clustering of probabilities f AMI and No-AMI groups than did the expert's in tation of the ECHO/EKG data. This highlights s important limitations in the study design. Most i tantly, ECHO/EKG data were available for only a the patients included in the study set. Furthermo: cause the data were collected retrospectively, the c of the ECHO/EKG data in some cases was margin. attempted to correct for this by arbitrarily using a probability of 0.5 for what the cardiologist consider agnostic of AMI. Because the echocardiogram cann tinguish the ventricular wall motion defects seen AMI from those of a healed infarction, the ECHO data were more likely to produce false-positive diaj of AMI.

Finally, the population of patients who unde ECHO/EKG studies may have been biased becau echocardiogram would be more likely to be orde cases in which the cardiac enzymes or EKG diverge the clinical impression. In fact, the network's thre diagnoses, which occurred in the ECHO/EKG { would have been misdiagnosed by the expert's crite CE interpretation. This finding diminishes the impo of the poor statistical correlation between the cardiol predicted probability of AMI based on the ECHO/ data and the neural network's probability based on CEs. It also serves to highlight the question of which aside from serial CEs, should be considered diagnos AMI in the living patient.

Autopsy material was reviewed, with the assum that the results represented the "gold standard" f agnosis of AMI. Only one misdiagnosis occurred autopsy group. One patient showed a total CK dropped from 1,110 to 772 U/L over a 48-hour int accompanied by a persistent 2% CKMB fraction, . total LDH that rose slightly from 264 to 280 U/L neural network misdiagnosed this as AMI, with a probability. The autopsy showed systemic amylo with severe myocardial involvement, resulting in cardial ischemia (fuchsinorrhagia) but no infarction network may have been "fooled" into a misdiagno the declining total CK, which was not mirrored by cline in the CKMB fraction, but its exact reason: obscure.

This highlights a rather fundamental limitatio neural networks. Unlike most rule-based systems, v allow the user to review the logic leading to a parti solution, neural networks cannot easily "explain" reasoning. At best, the user can review various neu weighting factors and connections. However, the a tectural complexity of even simple networks makes ficult to track an input vector beyond more than a s hidden layer of neurons. While this deficiency shoul

TABLE 3. REPRESENTATIVE EXAMPLES OF INPUT NEURON VECTORS AND RESULTING OUTPUT

Test	Example 1	Example 2	Example 3	Example 4
Total CK-1	728	49	20	45
% CKMM-1	89	100	100	100
% CKMB-1	11	0	0	0
% CKBB-1	0	0	0	0
Total LDH-1	635	1,430	607	722
% LDH1-1	36	32	25	16
% LDH2-1	31	33	33	26
% LDH3-1	17	18	22	23
% LDH4-1	8	7	10	12
% LDH5-1	8	10	10	23
Total CK-2	2,662	37	35	40
% CKMM-2	88	100	100	100
% CKMB-2	12	0	0	0
% CKBB-2	0	0	0	0
Total LDH-2	1,373	1,576	842	540
% LDH1-2	42	29	29	18
% LDH2-2	28	29	35	28
% LDH3-2	13	18	19	24
% LDH4-2	7	10	9	11
% LDH5-2	10	14	8	19
Δ hours	7.4	23.7	36.5	7.8
Network	0.999	0.887	0.110	0.000
Expert	1	0	0	0

Examples 2 and 3 represent the only paired CE sets for which the network calculated indeterminate probabilities.

Δ hours = time interval in hours between paired CE sets.

Network = Neural network's assessment of CE data shown, representing probability of AMI; Expert = human expert's assessment of all CE data from same patient (1 = AMI; 0 = No-AMI). Total CK and LDH values expressed as U/L.

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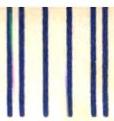
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vent a clinician's blind acceptance of a neural network's solution to a medical problem, it should not limit the network's utility within certain domains of problem solving.

To date, neural network applications in clinical medicine have been limited but, nonetheless, have been impressive.[14-17] The present level of technology would support neural network analysis of patient data related to a particular disease or, possibly, a family of diseases within one organ system. Hybrid designs, using expert systems to locate the disease or organ system of interest followed by neural network analysis of that small subset of the relevant patient data, are practical with existing technology.[18]

In this study we have demonstrated that a fairly simple neural network can be successfully trained to analyze serial CE data. The network's analyses agreed with those of an independent expert and were validated as diagnostically correct by autopsy. The application of neural network data analysis to other clinical tests may prove to be a useful diagnostic adjunct to traditional methods, either as stand-alone modules or as hybrids within larger expert systems.

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Original Article

The Opacity of Portal Hypertension-Related Ascites Correlates with the Fluid's Triglyceride Concentration

BRUCE A. RUNYON, M.D., EVANGELOS A. AKRIVIADIS, M.D.,
AND ANTHONY J. KEYSER, PH.D.

To determine if an elevated triglyceride concentration can explain the opacity of some cirrhotic ascites specimens, the authors measured triglyceride concentration by Coulter DACOS® (Hialeah, FL) on 133 paired serum and ascitic fluid specimens. The specimens were categorized as clear or cloudy by coded visual inspection. In addition, the ascitic fluid specimens were inspected for a lipid supernatant after 48 hours of refrigeration at 4 °C. The ascitic fluid triglyceride concentration of the 87 clear specimens was 1.9 ± 1.0 mmol/L compared with 7.0 ± 4.6 mmol/L for the opalescent specimens ($P < 0.001$). Only 17% of the clear specimens demonstrated any lipid layer after refrigeration, compared with 94% of opalescent specimens ($P < 0.001$). The triglyceride concentrations were not significantly different between the serum samples obtained from patients with clear compared with opalescent ascites. The opacity of portal hypertension-related ascites appears to be related to the triglyceride concentration of the fluid. (Key words: Ascitic fluid; Opalescent ascites) Am J Clin Pathol 1991;96:142-143

It is well known that the opacity of chylous ascites is due to a markedly elevated triglyceride concentration.[1,2] The explanation for the cloudiness of some cirrhotic ascites specimens that are acellular, but not frankly chylous, is unknown. The observation that the triglyceride concentration of some of these "opalescent" specimens is elevated prompted this study to determine if the explanation of the cloudiness of a large number of these specimens is elevated triglyceride concentration.

MATERIALS AND METHODS

Simultaneous ascitic fluid and serum specimens were obtained from patients admitted to the University of Southern California Liver Unit at Rancho Los Amigos Medical Center. Ascitic fluid was tested in the routine fashion for cell count, differential, and albumin concentration.[3-5] Cirrhotic (portal hypertension-related) ascites

was diagnosed when the serum-ascitic fluid albumin concentration gradient was ≥11 g/L and there was clinical evidence of chronic parenchymal liver disease.[3,6] Serum and ascitic fluid triglyceride concentration were measured on the Coulter DACOS® (Hialeah, FL) using a colorimetric enzymatic method. Ascitic fluid also was inspected visually in glass tubes in a coded fashion and categorized as "clear" or "cloudy" (*i.e.*, opalescent) based on the ability of the inspector to read print through the fluid. In addition, fluid samples were placed in a refrigerator in glass tubes at 4 °C for 48 hours, and the fluid was inspected for the presence of a lipid layer.

RESULTS

One hundred thirty-three paired serum and ascitic fluid specimens were tested. The mean triglyceride concentration of the 87 clear cirrhotic ascites specimens was 1.9 ± 1.0 mmol/L, compared with a value of 7.0 ± 4.6 mmol/L for the 46 cloudy specimens. This difference in triglyceride concentration was significant ($P < 0.001$). This difference did not appear to be explained by a difference in serum triglyceride concentration (mean serum triglyceride in patients with clear cirrhotic ascites = 9.7 ± 5.6 mmol/L, serum triglyceride of patients with cloudy ascites 10.7 ± 5.5 mmol/L, $P > 0.1$).

Fifteen (17%) of the 87 clear cirrhotic ascites specimens developed a lipid layer upon refrigeration. This was significantly different compared with the 43 (94%) of 46 cloudy specimens that demonstrated a lipid layer ($P < 0.001$).

DISCUSSION

The cloudiness of many ascitic fluid specimens is explained by an elevated white blood cell count, as in spontaneous bacterial peritonitis. However, a substantial number of specimens have normal cell counts but are cloudy. This study demonstrates that the opacity of such specimens is largely explained by an elevated triglyceride concentration.

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CORRESPONDENCE AND CORRECTIONS

Effect of Fixatives and Fixation Times on Tissues

To the Editor:—Greer and colleagues compared the effects of various fixatives and fixation times on the polymerase chain reaction-mediated amplification of DNA obtained from paraffin-embedded tissues.[1] They reported better results in tissues fixed in formaldehyde than those fixed in coagulating fixatives. Their results differ from those of previous workers who reported that alcohol-based fixatives are better than formaldehyde for the preservation of nucleic acids.[2-4] Furthermore, others have reported that the quality of extractable DNA and RNA is inversely proportional to the duration of formaldehyde fixation.[2,5-7]

It is noteworthy that Greer and colleagues found that the best amplification was observed on samples "fixed" in formaldehyde for only 1 or 4 hours.

The disparity of Greer's findings with those of previous researchers may be explained by critically considering the experimental design used by Greer and colleagues, in light of what is known about the chemistry of formaldehyde fixation. When formaldehyde is dissolved in water, it quickly hydrates to methylene glycol, a nonfixative, and only a small fraction (0.1%) of the dissolved formaldehyde remains as free formaldehyde, available to bind with aminoacids. As the scant available free aldehyde binds with tissue components, room is freed for additional methylene glycol molecules to dehydrate and release free formaldehyde, which in turn binds to more tissue components, and so on. This reaction, known to chemists as a "clock reaction," is slow and requires 24 to 48 hours to be complete.[8]

It follows therefore, that the tissues exposed to formaldehyde for 1 to 4 hours in Greer's study were incompletely fixed by the aldehyde. Fixation, rather, was largely provided by the alcohol used after the brief formalin exposure.

It is thus possible to reconcile the apparent difference between the results of Greer and co-workers and those of previous workers. In their study Greer and colleagues compared tissues exposed to formaldehyde for periods of 1, 4, and 24 hours, with tissues fixed for the same period of time in other fixatives, including alcohol-based fixatives. The best results

were obtained in tissues exposed to formalin for 1 to 4 hours and some deterioration was already evident at 24 hours. Thus I believe that Greer's best results were obtained in tissues essentially fixed in alcohol, and thus are in agreement with those of previous workers who compared formaldehyde with alcohol fixation.[2-4] Parenthetically, ethanol alone should have been the appropriate control in the Greer study.

Additional support for my interpretation is provided by Greer and her colleagues (note added in print), when she states "preliminary studies suggest that OmniFix (American Histology Reagent Co., Stockton, CA) may be superior to 10% neutral buffered formalin for subsequent amplification of paraffin-embedded tissues," because OmniFix does not contain aldehydes and is essentially an alcohol-based fixative.

In addition, it is important to emphasize that fixation times of 1 to 4 hours in formaldehyde are unrealistic and practically never used in the diagnostic histology laboratory. Furthermore, it is not uncommon to exceed the 24-hour fixation time (weekends, large specimens, and so on). Such prolonged exposures to formaldehyde are not only harmful to antigenic sites[9] but may also yield poorly preserved DNA and RNA.[2-7] In contrast, prolonged immersion of tissues in ethanol followed by paraffin embedding does not seem to noticeably damage proteins, DNA, and RNA.[2,3,9] Thus, plain ethanol may be a more appropriate fixative for DNA/RNA amplification studies.

I agree with Greer and associates that "a thorough review of the documentation regarding the processing of each sample when designing large retrospective studies" would be most useful. Unfortunately, most pathology laboratories have not observed strict control over fixation time, and few laboratories would be in a position to produce adequate documentation of this nature. Thus, Greer and co-workers' recommendation that a pilot study to evaluate the preservation of DNA on archival material be carried out before embarking on large retrospective studies is sound and practical advice.

For prospective studies, however, cur-

rent evidence suggests that no single fixative is ideal for the preservation of a wide range of molecules. It would then appear prudent, until a hypothetical universal fixative is developed, to fix tissues routinely in formaldehyde for periods not exceeding 24 hours, with an aliquot fixed in a mild coagulating fixative such as absolute ethanol, or OmniFix. Even better, a representative sample should be stored frozen at −70°C. This approach, which is routine in our laboratory, ensures the best possible preservation of morphologic detail, antigens, and nucleic acids.

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The Authors' Reply

To the Editor:—To reiterate, the purpose of our study of paraffin-embedded tissue sections was to evaluate several commonly used fixatives for their effect on subsequent polymerase chain reaction (PCR) DNA amplification. We compared 11 different fixatives at fixation times of 1, 4, and 24 hours. The effect of fixation was measure by the ability of the DNA in a prepared tissue to serve as a template for the amplification of DNA fragments of 110, 268, 536, 989, and 1,327 base pairs (bp) in length.

Dr. Battifora's letter cites previous work that he interprets to suggest that alcohol-based fixatives were superior to 10% buffered neutral formalin (BNF) for preservation of nucleic acids. Reference 3 (Battifora's letter) clearly shows that when fixed tissues were paraffin embedded (as in our study on which Dr. Battifora is commenting), in fact the greatest percentage of recoverable DNA was obtained from the formalin-fixed tissues, followed by ethanol-fixed tissues, and finally methanol-acetic-acid (MAA) fixed. Furthermore, the study (ref. 3) does not address PCR amplification, thus rendering it difficult to compare with our results. In addition, Dr. Battifora cites an abstract (ref 2) that states that for subsequent ampli-

fication, OmniFix™ (an alcohol-based fixative) and BNF were equivalent.

It is important to note that each of the alcohol-containing fixatives used in our study was also acid containing (low pH probably causes depurination and DNA strand scission during fixation), explaining the less efficient amplification from tissues processed in "alcohol-based" fixatives.

Despite the possibility that some large pieces of tissue may not be completely "fixed" after 24 hours in BNF, the results of our study remain clear and conclusive. After 24 hours of fixation, no other fixative (with the exception of acetone) evaluated in the study gave superior amplification results to those found with BNF. Thus, for routine biopsies, which are typically fixed for 24 hours or less, we reiterate our conclusion that BNF was the most desirable of the fixatives tested. In addition, we stress that for studies of suboptimally fixed or other "difficult" tissues, designing strategies that involve smaller DNA amplification products may prove useful.

Dr. Battifora's discussion of the advantages of ethanol fixation offers a nice prelude to the results from our long-term fixation study. This additional study addressed the effect of short- and long-term storage (2 hours to 30 days) in a variety

of fixatives (BNF, 95% ethanol, acetone, and OmniFix™) before paraffin embedding. We tested the ability of prepared tissue sections to yield DNA amplification products ranging from 268 to 1,327 bp. The results indicated that after 8 days, tissues fixed in BNF were able to amplify 536 bp, and even some but not 989 bp, DNA fragments; after 30 days of BNF fixation, only the 268-bp fragment was amplifiable. Samples fixed in OmniFix™ and acetone yielded products of 989 and 1327 bp, respectively, after 96 hours; both yielded 989-bp amplification products after 30 days of fixation. As might be expected, tissues fixed in 95% ethanol for up to 30 days efficiently produced DNA amplification fragments of up to 1327 bp in length.

These additional results provide useful information for prospective studies that involve eventual PCR from archival material. Furthermore, fixation and long-term storage in ethanol should prove particularly useful in remote areas where refrigeration or immediate sample processing is unavailable.

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B Cells and UCHL1 Antigen Expression

To the Editor:—I read with interest the correspondence on the nature of the paracortical CD30-positive blasts in infectious mononucleosis (IM).[1] The phenotype CD30+, Leu-22+ (CD43), UCHL1 weak+ (CD45R0) reported by Hodges and colleagues is by no means diagnostic of T-cell lineage. As acknowledged by Abbondanzo and associates,[2] CD30 antigen is not lineage specific and plasma cells and related neoplasms may be CD30 positive.[3] CD43 reagents, and especially Leu-22, stain both T and B cells and strong reactivity is reported in plasma cells, plasma cell neoplasms, and reactive plasmablasts.[4]

The question of whether UCHL1 can stain non-neoplastic B cells is more interesting. UCHL1 recognizes the p180 isoform of the leukocyte common molecule (LCA). T cells are known to switch from

the high (recognized by CD45RA) to the low molecular weight isoform (recognized by UCHL1) as they assume primed or memory function. It has been held that B cells do not switch, except for the complete loss of LCA in plasma cells. However, about 10% of high-grade B-cell lymphomas, some plasmacytomas, and myeloma cell lines are UCHL1 positive.[5,6] Notably, Epstein–Barr virus-related polymorphic immunoblastic lymphomas occuring in immunosuppressed patients show heterogeneous UCHL1 and CD45RA staining. This occurs principally in the plasmablasts, suggesting isoform switching as the cells mature to plasma cells.

In two cases of florid IM lymphadenopathy we have found groups of CD30+, CD43+, UCHL1 weak+, L26−, and strongly cytoplasmic Ig+ blast cells and confirmed this by double staining. The

loss of L26 (CD20) immunoreactivity is in keeping with terminal B-cell differentiation and suggests that UCHL1 antigen is transiently expressed by normal B cells as they mature to plasma cells.[7] Alternatively, but less likely, this may reflect aberrant LCA isoform switching in Epstein-Barr virus-infected B cells.

Before relying too strongly on a weak UCHL1 result, it would be helpful to perform Ig and polyclonal CD3 staining on paraffin sections to determine more accurately the nature of the CD30-positive cells in their case.

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The Author's Reply

Phenotype of Atypical CD30-Positive Cells in Infectious Mononucleosis

I appreciate the informative comments of Dr. Norton in response to our report of immunostaining patterns of atypical lymphoid cells in a patient with recurrent infectious mononucleosis.

While my colleagues and I stated that the weakly positive UCHL-1 reaction supported a T-cell phenotype, it was not our intention to imply absolute evidence for the same. Additional studies on the CD30-positive cells revealed an unequivocally negative pattern for IgM. In contrast, however, rare IgG-positive cells were identified, raising the possibility that a small number of atypical elements in this case are, in fact, B immunoblasts.

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REFERENCE

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Screening RIA Serum Samples

To the Editor:—Riccio and colleagues in their paper "The Effects of Radioisotopes Used in Nuclear Medicine on Diagnostic Radioimmunoassay Testing" (Am J Clin Pathol 1990;94:618–623) conclude that screening all radioimmunoassay patient serum samples is unnecessary, with the exception of Cobalt 57-based assay systems. They are correct and a simple screening method recently has been described by Plant.[1] She found radioisotope contamination of test serum in 22 of 7,000 samples. Potentially falsely low specimens were screened by merely placing them under a general purpose radiation monitor (model 425) with a thin crystal gamma radiation probe (Victoreen Nuclear Associates, Cleveland, OH). This screening method is simple, rapid, and inexpensive, and it does not require additional test serum.

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REFERENCE

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To the Editor:—Dr. Riccio and his associates[1] conclude in their study of radioisotope contamination of serum that the effect on diagnostic radioimmunoassays is not great enough to warrant screening all submitted sera for such contamination, with the tentatively stated exception of cobalamin (vitamin B12) assay. However, the problem is a complex one, and solutions ought to account for how the contamination actually affects the specific assay and its results.

For example, my study of serum cobalamin and folate radioisotope dilution assays showed that the impact of serum contamination on the assay results was great.[2] This was not because contamination was frequent; in fact, its overall incidence was probably as low as that found by Riccio and associates. The impact was great because radioactive contamination produced low results for "cobalamin" and "folate," and low results are clinically important. Thus, the effect of a relatively few contaminated sera was to produce a relatively large number of falsely low results; in my study, for example, 13 falsely low cobalamin results due to this artifact were found during the time that we encountered 26 truly cobalamin-deficient sera.[2]

Using a supernatant blank readily detected the artifact, but this is not a widely applicable solution because most assays no longer provide for such controls. A practical recommendation for cobalamin assay, therefore, would be to screen for endogenous radioactivity all sera found to give low results, rather than the extremes of either screening all submitted sera or screening none.

I would also note that the authors, who comment on the paucity of studies on the effect of serum radioactivity on diagnostic assays while citing only one abstract,

overlooked not only my paper, which appeared in this journal,[2] but also several others on this subject.[3,4]

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The Authors' Reply

To the editor:—We thank Dr. Wentworth for his comments and for bringing to our attention the recent publication by Sylvia Plant. The rapid method for determining preassay serum radiation contamination, with the model 425, thin crystal gamma radiation probe (Victoreen Nuclear Associates, Cleveland, OH), appears to be a reasonable alternative for rapid identification of these specimens. In our study we were able to screen 25 samples at one time using the Genesys counter as described in our paper. The finding by Plant that significantly erroneously low vitamin B_{12} measurements were obtained appears important. Although we had hoped that this would not be the case, as we noted in our study, we could not effectively differentiate contaminating gamma radiation from that of the cobalt

57 radioligand. This is due to the partial overlapping of energy levels of cobalt 57 and all the other contaminating radioisotopes.

It appears that we should continue to monitor for contaminating preassay radioactivity in the serum of patients with vitamin B_{12} or folate test ordered, and perhaps periodically recheck these serum samples for decay of the radionuclide to a point at which the test may be performed reliably. In most instances this delay probably would produce no clinical significance, except in the case of administration of radiopharmaceuticals with extremely long half-lives, such as cobalt 57 (270 days) and iodine 125 (60 days). In these cases, it may behoove the clinicians to draw these specimens before dosing with these long half-life radiopharmaceutical.

We also wish to thank Dr. Carmel for his comments concerning the difficulties related to contamination of the folate and cobalamin tests as outlined in his article. We regret having overlooked these important contributions, but we are pleased that our study, using a different method. independently raised the same concerns about this contamination in the B_{12}/folate assay.

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EDTA-Dependent Leukoagglutination

To the Editor:—Hillyer and Associates reported an excellent case of spurious leukopenia secondary to leukocyte aggregation caused by an ethylene dinitrilo tetraacetic acid (EDTA)-dependent IgM antibody.[1] I encountered a case in which mainly granulocytes were aggregated on the peripheral blood smear. The case involved a 62-year-old man admitted to King Faisal Specialist Hospital and Research Centre with the diagnosis of bronchopneumonia. A white blood cell count

(WBC) of 4.3×10^9/L was reported from an automated cell counter (Coulter S-Plus III) on blood sample collected in EDTA. Review of the peripheral blood smear for differential count drew our attention to the granulocyte aggregates of 20 to 30 cells each (Fig. 1). A blood sample collected in citrate anticoagulant showed WBC of 9.6×10^9/L (more than 50% WBC increase from the previous sample) and disappearance of the aggregates. A retrospective review of the previous complete blood cell

counts in the patient's chart revealed a continuous discrepancy of the WBC (*e.g* . on March 22, 1988 at 15:30 P.M., the WBC was 17.1×10^9/L compared to 9.5×10^9/L at 20:35 P.M.). From this case and other reported cases,[2,3] we concluded that this IgM antibody is directed specifically to a granulocyte antigen and depends on the presence of EDTA. Further studies on antibody specificity should be conducted. Although the condition seems to be very rare, many cases might have
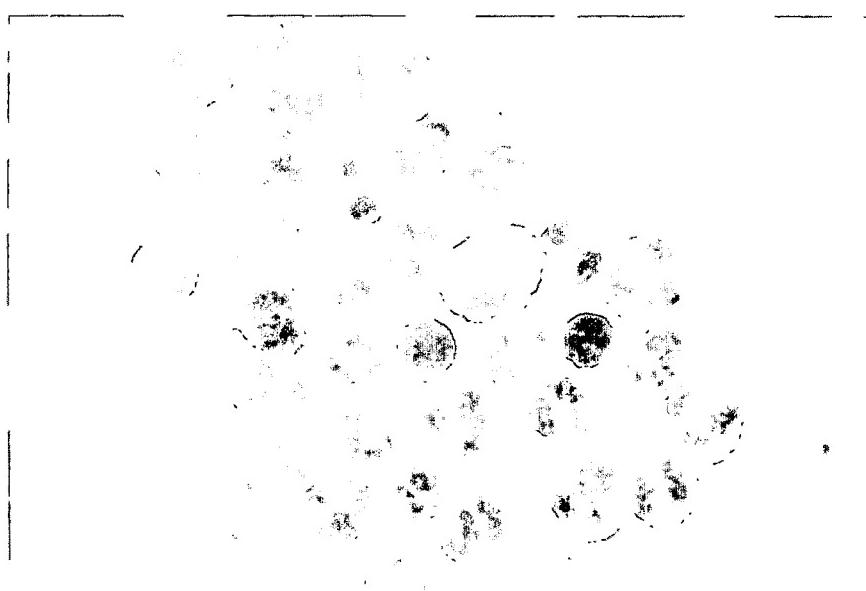
been missed or underestimated. If such artifact occurred in a case of CML on treatment, the doubling time and monitoring of chemotherapy could have been seriously affected.

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Thymic B Cells

To the Editor:—We read with interest the recent article by Eimoto and colleagues on "Nonneoplastic and Non-hyperplastic Thymus in Myasthenia Gravis".[1] Their study demonstrated that thymuses of patients with myasthenia gravis (MG), which appear normal by routine light microscopy, show changes of thymic microenvironment that can be detected by immunohistochemical examinations, especially double-labeling methods. They describe distension of the perivascular space (PVS) with elongation of medullary epithelium, disruption of basement membranes, and increased numbers of B cells within the PVS. In lymph follicular hyperplasia, the same architectural disturbances are present in a much more pronounced way, thus supporting the uniting concept of chronic thymitis.[2,3]

However, we are skeptical whether it is always possible to distinguish clearly between chronic thymitis in MG patients without lymph follicular hyperplasia (type A) and normal thymus. B cells, which play a prominent role in the immunopathologic state of MG, have only recently been described as a constant constituent of the normal thymic microenvironment.[4,5] Detailed information has been obtained on the immunophenotype and morphology of the intramedullary B-cell population, but relatively little attention has been given to the B cells of the "extrathymic" PVS and their variability, both in number and distribution in the normal thymus. Most authors regard them as passenger lymphocytes without functional relevance. We have examined a series of normal fetal, juvenile, and adult thymuses as well as nonneoplastic thymuses from myasthenic patients with single- and double-labeling immunohistochemical techniques, using both cryostat sections and routinely fixed, paraffin-embedded material.[6,7] The number of B lymphocytes in the PVS of thymuses from patients with-

out signs of autoimmune disease varied significantly, showing a parallel increase to the volume of the PVS and the occurrence of lymphoid follicles, which can be found in about one fourth of normal thymuses by serial sections.[8] In older patients with signs of thymic atrophy, the PVS is often expanded, with a subsequent relative increase in B cells. In accordance with other findings, we were always able to locate the lymphoid follicles in the PVS by serial sections and double-staining experiments, separated from the medulla by a row of epithelial cells.[4] However, the epithelial layer around these follicles showed discontinuities, and many B cells from the outer follicle zone seemed to penetrate into the adjacent medulla. The findings in some of the patients without MG resemble closely the alterations described by Eimoto and co-workers[1] in so-called type A thymus of MG patients. In our opinion, these moderate changes are not necessarily specific for autoimmune disease. We sup-

pose that various influences can stimulate the thymic B-cell system and lead to this form of "chronic thymitis" without lymphoid follicular hyperplasia, which may sometimes simply represent immunologic activation and not an immunopathologic state.

Until now, the role of the medullary B cells in MG and the interrelations between intrathymic and extrathymic B-cell compartment have received little attention. Although an influx from B cells from the PVS into the medulla in MG seems likely from histologic observations, the fate of the original intramedullary B-cell population, especially the large, CD23-positive cells with dendritic features, which represent a unique phenotype,[4] remains uncertain. Only an analysis of the function of these truly intrathymic B cells and of

their interactions with the peripheral B-cell population will yield a more complete view of the thymus in states of health and disease.

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The Authors' Reply

To the Editor:—We thank Dr. Fend and colleagues for their interesting comments. Their letter notes that (1) the number of B lymphocytes in the perivascular space (PVS) of "normal" thymuses is variable, (2) in older patients without myasthenia gravis (MG), lymphoid follicles in the PVS separated from discontinued epithelial layer can always be located, and thus, (3) the changes in nonneoplastic and nonhyperplastic thymus of myasthenic patients in our description may represent "simple immunologic activation, not an immunopathologic state." We are inclined to agree with some of their observations, but not with their interpretations.

As they indicate, B lymphocytes may be present in the PVS as well as in the medulla of non-MG thymuses. However, in our experience we have found the degree and frequency of their appearance in the PVS to be much less in non-MG thymuses compared with MG thymuses; only one (case 27) of eight age-matched control cases in our series contained a considerable number of B cells in the PVS.[1] Their finding of lymphoid follicles in the PVS of most thymuses of older nonmyasthenic patients is of interest, although we could not find such descriptions in reference

number 4 that they cited. If this finding is verified and their logic is followed, even lymph follicles in MG thymuses are not immunopathologic? Our first question to the interpretation by Dr. Fend and colleagues is whether these changes in non-MG thymuses are normal, as they mention at first. Later, they call the changes "immunologic activation." Our second question is how one can regard the immunologic activation in MG thymus as "not immunopathologic" when it is associated with production of anti-acetylcholine receptor antibody and/or anti-striated muscle antibody.[2]

The conclusion in our study was based not only on B cells but also on various other components. The changes we described represent an immunopathologic state (chronic thymitis in a broad sense) with immunologic activation on the basis of a significant architectural disturbance in the MG thymuses with or without follicular hyperplasia. The changes as shown in Figure 1A compared with Figure 1B in our article[1] hardly suggest a simple physiologic process. We do not think, however, that the morphologic changes in MG thymuses are specific for the disease. Chronic thymitis not associated with MG may exist as the occurrence of thymic follicular

hyperplasia is well known in other autoimmune diseases and in the absence of significant diseases.[3] A specific etiology and/or HLA genotype may underlie the development of morphologic changes in MG thymus,[2] although we concur with Dr. Fend and colleagues as to the importance of analyzing the intra- and extrathymic B-cell compartments.

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American Society of Clinical Pathologists/ College of American Pathologists

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The three-day conference will include sessions on historical information of interest, analysis of market needs for automated screening, challenges for specimen standardization, issues of quality assurance, the possible role of the Federal Food and Drug Administration in this area, discussion of commercial applications of currently available technology, and workshops offering small group demonstrations of pertinent equipment. Participation is open to physicians, medical technologists, cytotechnologists, laboratory managers, and other interested members of the healthcare industry. For further information contact: The International Institute for Applied CytoSciences, Inc., 40 Beach Street, Suite 304, Manchester, MA 01944, or call (508) 526-8083; or Heinz K. Grohs, M.D., F.I.A.C., Program Chairman, (508) 724-1200, ext. 4101.

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American Association of Pathologists

October 31–November 3, 1991   *Concepts in Molecular Biology* (Bethesda, Maryland)

Co-sponsored by the United States and Canadian Academy of Pathology and the College of American Pathologists, this course will consist of lectures, tutorials, laboratory demonstrations, and a syllabus with annotated bibliographies and laboratory protocols. The course has been designed for diagnostic and experimental pathologists, basic scientists, and clinical investigators who desire to become conversant with the basic principles and concepts of recent advances in biotechnology. Emphasis will be placed on understanding nucleic acid molecular biology and its application to diagnosis and pathogenesis of human disease. Space is limited, so register early. Category I CME credit will be available. For more information and registration forms contact: The American Association of Pathologists, 9650 Rockville Pike, Bethesda, MD 20814-3993; or call (301) 530-7130 or fax (301) 571-1879.

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Department of Pathology, Massachusetts General Hospital, Harvard Medical School

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Pathology, University of Utah School of Medicine, Salt Lake City, Utah 84132; (801) 581-7480.

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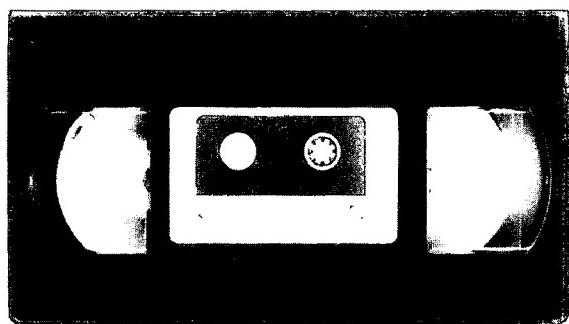
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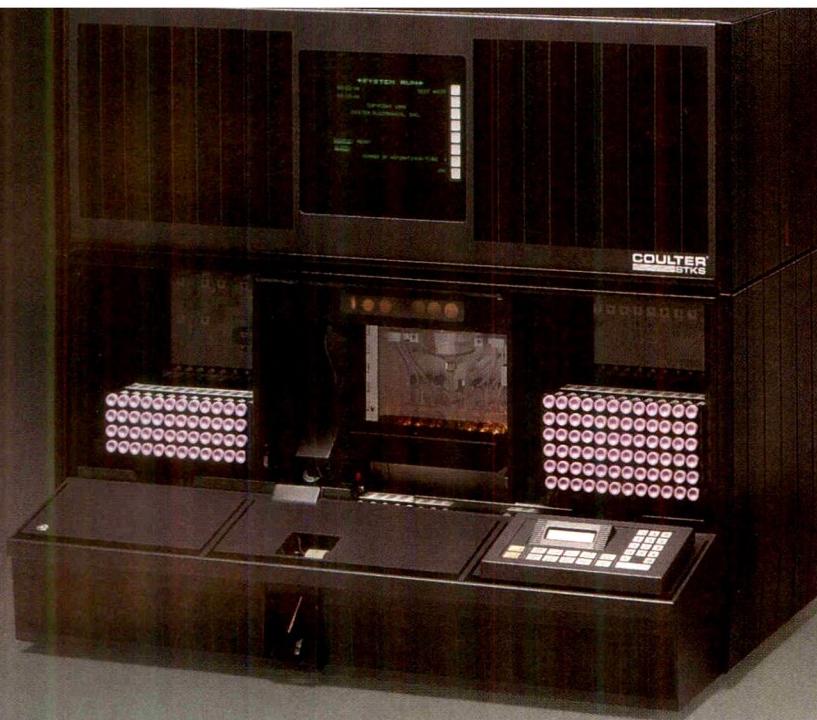
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Evolution of the French-American-British (FAB) Proposals

Is There a Place for Acute Basophilic Leukemia?

In 1976 when the French-American-British (FAB) proposals were first published, categorization of acute leukemia seemed simple.[1] This disease was diagnosed and subclassified based primarily on Wright's stain morphology, with ancillary help from myeloperoxidase (MPO) and nonspecific esterase stains. There were two basic premises of the FAB proposals. First was to develop a system that "would permit comparison between series of cases, show prognostic differences between different categories, and provide a framework of reference for persons working in different biologic disciplines who use material from patients with leukemia."[2] Second was to "formulate diagnostic guidelines from cytologic material likely to be available readily in most hematology laboratories, namely, Romanowsky-stained films . . . and a few essential cytochemical reactions."[2]

In the 15 years since the original proposal, a wealth of new information from cytogenetics, immunology, and electron microscopy (EM) has been added to our understanding of acute leukemia.[3-11] With the acquisition of this new technology, the morphologic classification of acute leukemia can be validated further. Moreover, an expansion of such classification by technologic means must be considered.[12,13]

The report of eight cases of well-characterized acute basophilic leukemia by Peterson and co-workers, in the current issue of the AJCP (pages 160-170) adds further to the complexity of this issue. Where do these cases fit into the FAB classification? Is a new subtype (M8 or M2Baso) needed to accommodate them? When are electron microscopy and other technologies necessary in the "morphologic" classification of acute leukemia? The following brief outline of the FAB subtypes, as described in 1976, and modified in 1981, 1982, and 1985, will serve as a basis for discussing these questions.[1,2,14-16]

OUTLINE OF FAB SUBTYPES

Acute Lymphoblastic Leukemia (L1-L3)

Based on morphology, immunology, and cytogenetics, L3 acute lymphoblastic leukemia (ALL) is clearly distinct from L1 and L2, which form a morphologic spectrum.[4] L2 is less differentiated than L1, requiring cytochemistry to distinguish it from myeloid leukemia: terminal transferase and immunologic markers are used to confirm its lymphoid nature.[4] It is of interest that when the FAB clas-

sification was initially proposed, the diagnosis of some cases of L2 ALL was made by exclusion of myeloid leukemia with the MPO stain, because B-cell and myeloid-specific antibodies were not in general use.

Acute Myeloid Leukemia (M1-M6)

Acute myeloid leukemia (AML) is divided into six subgroups (M1-M6), as defined by the original classification. M1, M2, and M3 are granulocytic in origin: M5a and M5b are monocytic: M4 is a hybrid of granulocytic and monocytic leukemia: M6 shows erythroid predominance.

Of the granulocytic leukemias, M1 shows little morphologic evidence of differentiation to granulocytes and may appear undifferentiated. It is considered granulocytic (myeloblastic without maturation) because of reactivity with the MPO stain and is separated from M2 (myeloblastic with maturation) by finding less than 10% of cells at the promyelocyte stage and beyond. There is good evidence, from cytogenetics and immunology, that hypergranular M3 and its microgranular variant (M3v) are distinct from M1 and M2.[10]

Other Cell Lines and Morphologically Undifferentiated Leukemia

It is not apparent from the original FAB proposals as to where rare cases with predominant maturation to basophils and megakaryocytes fit into the classification. It was stated in the 1976 FAB paper that "in rare cases. almost all of the myelocytes, metamyelocytes, and mature granulocytes may be eosinophils."[1] Eosinophils also may be a component of a subset of M4 with a chromosome 16 abnormality (M4Eo).[2,10]

It also was not made evident in 1976 proposals as to how cases with a predominance of undifferentiated blasts should be categorized. Thus, acute leukemia with undifferentiated blasts and negative myeloid cytochemical reactions appeared to "become" L2 ALL by default.[1] Currently, it is apparent that cases with undifferentiated blasts may be recognizable as (1) lymphoblastic or myeloblastic in nature, using immunologic markers or MPO activity on EM[5-8,12]; (2) megakaryoblastic in nature, based on immunologic markers or platelet peroxidase reactions by EM[3,12,15]; (3) basophilic in derivation, as determined by electron microscopy (see Peterson and co-workers); or (4)

biphenotypic, as judged by immunologic or ultrastructural evidence of both lymphoid and myeloid differentiation.[6,8,9,12]

Where do cases with megakaryocytic, "minimal myeloblastic," and biphenotypic differentiation fit into the FAB classification?

M7 (Megakaryoblastic)

Cases with megakaryocytic differentiation (M7) have now been added to the original six FAB subtypes.[16] This seems plausible, because in the original proposals, M1-3 leukemias are granulocytic, M5 AML is monocytic, and M6 leukemia is erythroid. Some cases of megakaryoblastic leukemia (M7) show maturation to atypical megakaryocytes. However, other examples of M7 are morphologically undifferentiated and must be studied with electron microscopy for platelet peroxidase activity or monoclonal antibodies to define them as such.[3,16]

Early Myeloblastic (M0)

The FAB group has not yet adopted "M0" as an official subtype, although this designation has appeared in the literature.[7,12] Cases that are so defined are morphologically undifferentiated and are negative with the MPO stain. They are identified as myeloid by the presence of appropriate surface antigens, or demonstration of MPO by electron microscopy or anti-MPO antibodies.[5-8] If these leukemias were officially defined as M0, they would add a fourth FAB subtype to those designated as having granulocytic differentiation (*i.e.,* M0, M1, M2, and M31).

Biphenotypic Acute Leukemia

A need also exists to define precisely a place in the FAB classification for cases in the literature that have been designated as biphenotypic.[6,8,9,12] Many of these are clearly lymphoid or myeloid by morphology and cytochemistry, and fit easily into one of the FAB subtypes. Nonetheless, they may show antigenic expression or ultrastructural evidence of both lymphoid and myeloid features. A smaller set of biphenotypic cases are morphologically undifferentiated or ambiguous, and rare examples appear to represent a dual proliferation of distinct myeloid and lymphoid cell lines. The latter two groups may deserve a separate designation within the FAB proposals.[12]

ACUTE BASOPHILIC LEUKEMIA

With this brief outline of the FAB classification as a background, we will now examine the eight cases of acute basophilic leukemia reported by Peterson and associates. These neoplasms were somewhat heterogeneous in terms of morphology, immunology, and chromosome altera-

tions. Two showed evidence on Wright's stain of maturation to basophils (cases 1 and 7). The remaining six lesions appeared to be morphologically undifferentiated, with the exception of a few "azurophilic or deep blue granules." In all cases, basophil or mast cell differentiation was evident after ultrastructural examination.

Where do these eight proliferations belong in the FAB classification? Should they be considered as granulocytic and placed in the categories of M0, M1, or M2, or do they deserve a separate designation of their own (M8)?[12]

A case can be made for establishing a new category of M8 AML for acute basophilic leukemia, rather than "M2Baso," as recently suggested by Catovsky and associates.[12] There is evidence that eosinophils and basophils have a common stem cell, and that basophils and mast cells may be derived from a marrow stem cell that is distinct from neutrophils.[17-19] Thus, from a functional standpoint, it may be reasonable to assign acute basophilic and eosinophilic leukemias their own M designations, as is true of megakaryocytic (M7) leukemia. One could reserve the designation "M2Baso" for those cases of M2 AML with neutrophilic differentiation and an associated increase in basophils, in analogy to M4Eo. If M2Baso is selected as an appropriate designation, only two of the eight cases described by Peterson and associates would meet the criteria for this entity: namely, those that showed definite conventional morphologic differentiation to basophils. Would the case that showed only ultrastructural cytologic evidence of basophil differentiation, but was also MPO positive, be classified as an "M1Baso"? Would the four cases with a negative MPO stain be classified as "M0Baso"?

The above questions cannot be answered by this commentator. Nevertheless, well-defined and consistent placement of acute leukemias with basophilic differentiation is needed in the FAB proposals to facilitate meaningful communication between different investigators.

It is of interest that among 46 well-characterized cases of lymphoid antigen-negative, acute, undifferentiated leukemia from three separate studies, basophil or mast cell granules were not observed by ultrastructural analysis.[5,7,8] This fact implies that the findings reported by Peterson and co-workers may be extremely rare. Alternate explanations might be that the granules were not recognized in other ultrastructural studies or that cases were excluded if such cellular organelles were observed.

WHEN ARE SPECIAL STUDIES NEEDED?

How often must immunology, cytogenetics, and electron microscopy be employed in the "morphologic diagnosis" of acute leukemia? Table 1 summarizes those subtypes that can be comfortably diagnosed morpholog-

Editorial

TABLE 1. SPECIAL STUDIES IN THE MORPHOLOGIC (FAB) CLASSIFICATION OF ACUTE LEUKEMIA

Morphologically Differentiated		Poorly Differentiated or Undifferentiated	
Morphologic Exam Only	Special Stains or Immunology Are Confirmatory	Special Stains Critical to Diagnosis	Immunology or Electron Microscopy Critical to Diagnosis
M2	NSE	MPO	Immuno
M3	M4	M1	Some L2
M6	M4Eo	M3v	
Acute Base*	M5b		Immuno and/or EM
		NSE	M0
	Immuno	M5a	M7[+]
	L1 and some L2		Biphenotypic
	L3		
	M7*		EM
			Acute Base[+]

* With maturation
[+] Without maturation.
NSE = nonspecific esterase.
MPO = Myeloperoxidase stain.
Immuno = Immunologic studies.
EM = Electron microscopy.

ically, and those that require additional special studies. The appearances of the processes in the two left columns of Table 1 are sufficiently characteristic that special stains and immunology are not critical, but may be useful, in the morphologic diagnosis using FAB proposals. Of course, it is now evident that special analyses, such as immunology, cytogenetics, and (possibly) molecular techniques provide additional prognostic or therapeutic information beyond that obtained from strict morphologic classification of several subtypes of acute leukemia.[4,10,12,13] Cytogenetics also may be helpful in confirming the nature of some cases of M3 and M3v.[10] The proliferations in the far right column of Table 1 are morphologically undifferentiated or poorly differentiated, and thus clearly require special studies that go beyond the original FAB proposals to establish a "morphologic" diagnosis. These leukemias also are relatively uncommon. Because immunology and electron microscopy are not available in all hematology laboratories for the diagnosis and therapy of acute leukemia, these cases should be referred to institutions with the necessary facilities to study them thoroughly. This step would optimize the procurement of new information pertaining to the pathobiology of hematopoietic malignancies.

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Lymphoepithelioma-like Carcinoma and Its Relationship to Epstein–Barr Virus

In 1921, Regaud and Schminke independently described a neoplasm occurring in the nasopharynx, characterized by anaplastic cells surrounded by many lymphoid cells. This tumor has come to be known as "lymphoepithelioma."[1,2] The nuclear features of the neoplastic cells in this lesion are distinctive, with a large, vesicular nucleus containing a single, prominent nucleolus. Although the cytoplasmic features are variable, the cellular borders often are indistinct, imparting a syncytial character to the neoplasm. The tumor cells may be arranged in nests (Regaud pattern) or as isolated cells (Schmincke pattern), but a definitional characteristic of the tumor is the presence of an intense lymphoid infiltrate, which has been characterized as primarily T-cell in nature. Light microscopic, electron microscopic, and immunologic studies of this neoplasm have confirmed its epithelial origin, and the term "lymphoepithelioma" gradually is being replaced by the designation of "undifferentiated carcinoma with intense lymphoid stroma." It is classified by the World Health Organization, along with squamous cell carcinoma and nonkeratinizing carcinoma (which lack a prominent lymphoid infiltrate and often possess desmoplasia), as a subtype of nasopharyngeal carcinoma.[3] Lymphoepithelioma (LE) comprises the largest category of neoplasms within the spectrum of nasopharyngeal carcinoma, tends to occur at an earlier age, favors Asian populations, is generally more radioresponsive, and possibly has a higher survival rate than the other types of nasopharyngeal carcinoma.

Epstein–Barr virus (EBV) was first identified in 1964 in cultured Burkitt's lymphoma cells and has since been associated with a wide variety of malignant lymphomas. These include B-cell lymphoma, T-cell lymphoma, and Hodgkin's disease, as reviewed elsewhere.[4] A specific relationship between LE and EBV was first suspected on the basis of serologic studies.[5] For example, high titers of antibodies against EBV are found in patients with LE, and a correlation is evident between the level of titer and tumor burden. Gaffey and Weiss[5] and Zur Hausen and others[6] identified EBV-DNA in a higher percentage of tumors by nucleic acid hybridization studies, and others have localized EBV nuclear antigen to the carcinoma cells. Subsequently, we[7] and others[8] demonstrated EBV nucleic acids within the neoplastic cells, but not in adjacent lymphocytes. LEs from all analyzed ethnic groups, including Asians, Caucasians, and blacks, have been found to contain EBV. Interestingly, nonkeratinizing carcinoma of the nasopharynx also has been shown to harbor EBV genomes.[8]

Carcinomas with morphologic features that are identical to those of LE only rarely occur outside of the nasopharynx, usually being seen in locations derived from the embryonic foregut. LE-like carcinomas have been reported in the tonsil,[9] salivary gland,[10-12] thymus,[13] lung,[14] and stomach.[15-17] These tumors are particularly uncommon in tissues that are not derived from the foregut, such as the skin[18] and uterine cervix.[19] Given the association between EBV and nasopharyngeal LE, many of these LE-like carcinomas also have been studied for the presence of EBV. LE-like carcinoma of the salivary gland ("malignant lymphoepithlial lesion") is a rare tumor with a predilection in Eskimos (justifying the synonym "eskimoma") and natives of Southern China. In 1982, Saemundsen and colleagues[10] reported the first salivary glandular LE-like carcinoma associated with EBV, identifying EBV genomes in an "undifferentiated" carcinoma from a Greenland Eskimo. Another group has confirmed these findings.[12] It should be noted that Eskimos also have a high incidence of LE of the nasopharynx, which occurs at a rate more than 15 times that of white persons in this context.

Since the latter report, other cases of EBV-positive LE-like carcinomas have been described in other organs. For example, Leyvraz and colleagues[13] reported a thymic LE-like carcinoma, in a Hispanic patient, which expressed EBV-associated nuclear antigen and contained several copies of viral genome. Butler and colleagues[14] described a case of pulmonary LE-like carcinoma in an Asian patient that similarly contained EBV genomes, as seen by *in situ* hybridization. Nonetheless, it appears that not all LE-like carcinomas harbor EBV genomes. We examined a series of 6 LEs of the nasopharynx and 14 LE-like carcinomas occurring in other organs, with a sensitive *in situ* hybridization technique.[7] Although we were able to demonstrate EBV genomes in all 6 cases of LE of the nasopharynx, we found EBV in only one LE-like carcinoma (the LE-like carcinoma of the lung described above, from Butler's series[14]). Other LE-like carcinomas of the skin, cervix,

palatine tonsil, floor of the mouth, and lung, all occurring in white persons were negative for EBV.

In 1990, Burke and associated[15] reported the first case of LE-like carcinoma that arose in the stomach. The tumor occurred in a 71-year-old Filipino man and was a 3-cm mass with serosal extension and metastasis to local lymph nodes and the liver. Histologically, the lesion resembled LE of the nasopharynx, without discernible glands or tubules. EBV was detected in that tumor with the polymerase chain reaction, but it was absent in a conventional poorly differentiated gastric adenocarcinoma that also had a pronounced lymphoid reaction. In the current issue of the AJCP, Min and colleagues[16] report three additional cases of LE-like carcinoma of the stomach. These neoplasms microscopically resembled LE of the nasopharynx, although one case showed foci of gland formation in the superficial portion of the tumor that suggested a relationship to poorly differentiated adenocarcinoma. In likeness to the case of Burke and associates,[15] all three neoplasms were positive for EBV genomes using the polymerase chain reaction.

In the study by Min and co-workers, *in situ* hybridization studies were performed but were reported as equivocal or negative. Thus, the cellular localization of the EBV in such cases is unclear. Min and colleagues suggested that *in situ* hybridization studies may be insufficiently sensitive to detect EBV genomes in these neoplasms. However, they used an *in situ* hybridization method that is based on a nonisotopically labeled probe, which, in our hands, has been found to be relatively insensitive for the detection of EBV genomes. It is notable that Min and colleagues did not use positive-control tissue (such as an EBV-positive LE of the nasopharynx) to assess the sensitivity of their technique.

We recently studied a series of eight gastric LE-like carcinomas, including seven from Japanese patients, using the polymerase chain reaction and *in situ* hybridization.[17] Seven of these cases contained detectable EBV genomes, as seen with the polymerase chain reaction. Six of the positive cases (including one lesion from a white patient also were studied by *in situ* hybridization. With ^{35}S-radiolabeled probes, EBV was detected in the tumor cells in each case, but it was not present in the reactive lymphoid infiltrate. The quantity of EBV present per tumor cell was roughly similar to that seen in positive cases of nasopharyngeal LE, as evaluated with an identical technique in a previous study. Thus, it does appear that the EBV is associated directly with this rare gastric neoplasm.

At the present time, one can only speculate on the role of EBV in the pathogenesis of LE of the nasopharynx and LE-like carcinomas in other organs. Our *in situ* hybridization studies have shown a relatively homogeneous distribution of EBV in all tumor cells, both in primary lesions and their metastases. Furthermore, analysis of the terminal region of the EBV genome in nasopharyngeal LE has revealed a single EBV episomal population in any given case. This provides evidence that this tumor (in likeness to most EBV-associated lymphomas) probably represents a monoclonal expansion of a single EBV-infected progenitor cell and not a "passenger" infection that manifests after the tumor has become established.[20]

Any model explaining the role of EBV in these neoplasms must address their peculiar geographic and racial predilections and anatomic locations. As mentioned above, nasopharyngeal LE is more common in Asia than in Western countries. It is also possible that thymomas occurring in Asia, in contrast to those seen in the United States may be associated with EBV. Nonetheless, confirmatory *in situ* hybridization studies on thymic tumors have not yet been reported. Environmental factors that are geographically related to this distribution may include exposure to tumor promotors such as croton oil and tung oil. These substances are found in trees endemic to Southern China. On the other hand, the predilection may be a racial one, because both LE and LE-like carcinomas appear to arise more commonly in Asian people. It is conceivable that Asians may have more EBV receptors on epithelial cells, making infection of such cells more likely. To date, EBV-positive LE and LE-like carcinomas have been identified only in the nasopharynx, salivary gland, lung, thymus, and stomach, all of which are foregut-derived structures. EBV normally infects pharyngeal epithelial cells and is often found in salivary gland tissue. Hence, the presence of EBV-positive tumors in the nasopharynx and the salivary gland is not surprising. However, it is difficult to envision direct spread of the virus from those sites to the thymus. It is possible, that EBV is carried in latently infected B lymphocytes to this organ because a small resident population of B cells normally is present in the thymus. Infection of epithelial cells in the lung or stomach conceivably could occur through contiguous spread or viral carriage in lymphocytes. It is interesting to note that a low level of EBV was identified in each of three gastric adenocarcinomas studied by Min and associates using the polymerase chain reaction. It may be possible that the gastric epithelium also is a reservoir for EBV. Alternatively, the positivity could have been derived from EBV-positive lymphocytes.

One final question centers on why EBV is associated with a tumor having a specific morphologic pattern—anaplastic cells arranged in a syncytial pattern with an exuberant lymphocytic infiltrate. The lymphoid reaction could be a direct response to the virus, or, more likely, to virally induced antigens expressed on the neoplastic cells. Several viral proteins are detectable on latently infected cells, including a membrane constituent that probably

represents a target for cytotoxic host T cells. When EBV infects lymphocytes, blastic transformation often occurs. Perhaps the anaplastic appearance of LE cells represents an equivalent phenomenon in epithelial cells. Nevertheless, these and other speculations must await additional studies of both EBV and virus-associated tumors.

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Acute Basophilic Leukemia
A Clinical, Morphologic, and Cytogenetic Study of Eight Cases

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The authors describe eight cases of acute basophilic leukemia. In six of the eight cases, basophilic involvement was not apparent by light microscopic examination. The cases were identified on the basis of ultrastructural evidence for basophil/mast cell differentiation of the blasts with little or no differentiation into other lineages. Ultrastructural analysis revealed immature basophil granules in blasts in all eight cases and theta granules in blasts in four cases. In three cases, ultrastructural evidence of mast cell differentiation also was present, with rare cells showing evidence for both basophil and mast cell differentiation. No clinical features distinguished this group of patients from others with acute myeloid leukemia. Cytogenetically, the cases were heterogeneous. Three had a Philadelphia chromosome; none had a t(6;9). The authors conclude that ultrastructural analysis usually must be used to diagnose acute basophilic leukemia, that acute basophilic leukemia is associated frequently with the Philadelphia chromosome, and that the ultrastructural findings provide evidence for a common origin of basophils and mast cells. (Key words: Basophilic leukemia; Basophils; Acute myeloid leukemia; Philadelphia chromosome; Mast cells) Am J Clin Pathol 1991;96:160-170

Increased numbers of basophils are characteristic of certain types of leukemia. Basophils are increased in the chronic phase of chronic myeloid leukemia and may become more prominent with disease progression, including blast crisis.[1] An increase in the number of basophils also has been found in a subset of acute myeloid leukemia that is marked by a chromosomal translocation involving the short arm of chromosome 6 and the long arm of chromosome 9: t(6;9)(p23;q14).[2] Basophilia in acute myeloid leukemia also has been associated with abnormalities of the short arm of chromosome 12.[3] In these instances, the increase in basophils represents only a small component of the leukemic process. Leukemias that involve the basophil lineage exclusively are rare[4] and are not well characterized.

In this report, we describe eight patients with acute basophil/mast cell leukemia and conclude that this type of leukemia usually requires ultrastructural analysis for diagnosis, frequently is associated with the presence of the Philadelphia chromosome, and provides evidence for a common origin of basophils and mast cells.

MATERIALS AND METHODS

The cases for this study were selected from 455 patients who presented with acute leukemia and had ultrastructural analysis performed on their leukemic blasts. Eight cases were identified as acute basophilic leukemia on the basis of ultrastructural evidence of basophil/mast cell differentiation of the blasts with little or no differentiation into other lineages. The cases also were examined by light microscopy and remained in the study only if the morphologic findings confirmed the basophil differentiation observed ultrastructurally or if the blasts appeared undifferentiated. Patients with a previous history of chronic myeloid leukemia, Down's syndrome, or systemic mastocytosis were excluded from the study, as were those with evidence of a panmyelopathy.

In all eight cases, Romanowsky-stained pretreatment bone marrow aspirate smears were examined. In seven

cases, blood smears and core biopsy specimens also were examined. Available cytochemical studies included myeloperoxidase (eight cases), Sudan Black B (seven cases), periodic acid-Schiff (seven cases), toluidine blue (six cases), and chloroacetate esterase (two cases). The blasts in seven cases were tested for terminal deoxynucleotidyl transferase (TdT) by an immunofluorescence technique using a rabbit anti-TdT antibody (Supertech, Bethesda, MD).[5] In addition, a cervical lymph node biopsy specimen from one case was reviewed; studies performed on this biopsy specimen included chloroacetate esterase and toluidine blue stains and immunostaining for lysozyme, alpha-1-antichymotrypsin, and Leu-M1.

Immunophenotypic analysis was performed on blood or bone marrow in three cases by flow cytometry and on frozen sections of a lymph node in one case, with the use of methods described previously.[6–8] In one case, the immunoalkaline phosphatase technique was used to immunophenotype a blood smear.[9]

Electron microscopic studies were performed on buffy coats from blood in three cases, bone marrow in three cases, and blood and bone marrow in two cases. In one case, a lymph node biopsy specimen also was examined electron microscopically. Fresh blood, bone marrow, and lymph node were processed for routine electron microscopic examination according to methods described previously.[10] Ultrastructural localization of peroxidase was performed in four cases. Cells were fixed for 30 minutes in 0.5% buffered glutaraldehyde at 0 °C and then incubated for one hour at room temperature in a reaction solution containing 2 mg/mL diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide. Specimens then were postfixed in 1% buffered osmium tetroxide and processed routinely for electron microscopic examination.

Identification of basophil and mast cell granules by electron microscopic examination was based on their distinctive ultrastructural appearance.[11,12] Mature basophil granules are membrane bound, are 0.1–0.4 μm in diameter, and contain an orderly arrangement of granulofilamentous material. The mature granules have an overall speckled appearance. Frequently, the water-soluble basophil granule contents are extracted partially during specimen preparation, leaving membrane-bound, clear, vacuolar structures. Many granules also contain electron-dense myelin figures. Immature basophil granules resemble mature granules except that the granular contents are not as electron dense or as tightly packed. Theta granules, which have been associated with very early basophil differentiation,[10,13] are membrane bound, 0.1–0.2 μm in diameter, and internally bisected by one or more electron-dense membranes. Mast cell granules contain electron-dense crystalline material arranged in a pattern of scrolls and lamellae, with an internal periodicity of approxi-

mately 7 μm. Glycogen-like particles are often interspersed between the scrolls and lamellae. Immature mast cell granules contain pale, amorphous material and loosely packed primitive scrolls and lamellae.

Cytogenetic studies of fresh heparinized bone marrow were performed in seven cases, according to methods described previously.[14] In the remaining case, blood was examined. Slides were made from direct preparations, 24-hour unstimulated cultures, and 24-hour methotrexate-synchronized cultures. Chromosome spreads were G-banded with the use of the Wright's technique of Sanchez and associates.[15] Metaphase spreads were analyzed fully at the microscope. Multiple photographs were taken on high-contrast film, and karyotypes were constructed in the usual fashion. Karyotypes were designated according to the International System for Human Cytogenetic Nomenclature (1985),[16] and abnormal clones were defined as specified at the Second International Workshop on Chromosomes in Leukemia.[17]

RESULTS

Patient Characteristics

Five patients were male, and three were female. Their ages ranged from 9 to 82 years; two patients were younger than 16 years old. Table 1 summarizes the clinical features, including presenting symptoms and physical findings, in the eight cases. Seven patients were treated with intensive chemotherapy (one received supportive therapy only). Of the seven treated patients, one underwent a bone marrow transplant. Four patients entered complete remission, two did not achieve remission, and one was lost to follow-up. Survival lengths ranged from 1 to 63+ months.

Laboratory Parameters

The laboratory parameters are summarized in Table 2. The leukocyte counts ranged from 2.8 to 144 $\times$ 10^9/L. All patients except one were anemic, and all were thrombocytopenic. Bone marrow blast percentages ranged from 33 to 99%. Three patients had peripheral basophilia, and three had marrow basophilia.

Light Microscopy

The light microscopic appearance of the blasts and the degree of maturation varied considerably from case to case. The nuclear contours of the blasts varied from round or oval to lobulated or deeply indented. Nucleoli often were prominent. The cytoplasm was scant to moderate and lightly basophilic. In one case, many of the blasts contained cytoplasmic vacuoles. There were no Auer rods in any of the cases. In three cases (cases 5, 6, and 8), the

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TABLE 1. CLINICAL FEATURES IN EIGHT CASES OF ACUTE BASOPHILIC LEUKEMIA

Case No.	Age/Sex	Presenting Symptoms	Physical Findings	Therapy	Outcome
1	9/F	Weakness, bruising	Hepatosplenomegaly	Adriamycin®, cytosine arabinoside	No remission Survival 54 days
2	13/M	Fever, sore throat, cough	Orbital edema, cervical lymphadenopathy, splenomegaly	Cytosine arabinoside and daunorubicin (2 courses), whole-brain irradiation, 5-azacytidine	Remission obtained Survival 63+ months
3	82/M	Night sweats, bruising	Hepatomegaly	Supportive	Survival 1 month
4	28/M	Fever, fatigue	Cervical lymphadenopathy, mediastinal mass	Cytosine arabinoside, 6-thioguanine, prednisone, daunorubicin, vincristine, L-asparaginase	No remission. Bone marrow transplantation done. Died at 21 months of graft-*versus*-host and residual leukemia.
5	51/M	Fatigue, cough, back pain	Hepatosplenomegaly	Daunorubicin, cytosine arabinoside, 6-thioguanine, vincristine, prednisone	Obtained remission in 2 months. Had a relapse. Second remission with cytosine arabinoside and L-asparaginase. Had a relapse and died at 26 months.
6	35/M	Malaise, headache, fever, chills, diarrhea	No organomegaly	Daunorubicin, cytosine arabinoside, 6-thioguanine, intrathecal methotrexate, prednisone	Remission in 5 weeks. Had a relapse 4 months later. Chemotherapy repeated. Remission achieved but patient died at 7 months of infection.
7	26/F	Bruising, gingival bleeding	No organomegaly	Cytosine arabinoside, daunorubicin	Remission achieved after 4 months. Lost to follow-up.
8	31/F	36 weeks pregnant, bruising, epistaxis, fatigability	Splenomegaly	Adriamycin, vincristine, prednisone, intrathecal methotrexate, cranial irradiation	Remission in 4 weeks. Had a relapse in 1 week. Remission in 2 months after L-asparaginase, intravenous methotrexate, cytosine arabinoside, etoposide. Had a relapse in 1 month. No remission achieved after high-dose methotrexate with leucovorin rescue and etoposide. Died at 11 months.

blasts were agranular and there was no evidence of basophil differentiation (Fig. 1). Mature basophils were rare or absent in the bone marrow and blood in these examples. In three cases (cases 2, 3, and 4), a rare or occasional blast contained several cytoplasmic granules that appeared azurophilic or deep blue (Fig. 2). Morphologically, some of these granules resembled those of mature basophils;

however, mature basophils either were not apparent or there were only a few in the specimen, constituting 0–1.6% of the total nucleated bone marrow cells. In two other cases (cases 1 and 7), occasional blasts with basophilic granulation were accompanied by cells showing differentiation into mature basophils/mast cells (Fig. 3). In these two cases, basophils (mature and immature, ex-

TABLE 2. HEMATOLOGIC PARAMETERS IN EIGHT CASES OF ACUTE BASOPHILIC LEUKEMIA

Case No.	Hemoglobin g/dL	Platelet Count ×10⁹/L	Leukocyte Count ×10⁹/L	% Basophils Blood	% Basophils Marrow	% Blasts Marrow
1	5.3	16.0	8.3	8	19	64
2	7.7	64.0	144.0	0	0	80
3	8.7	13.0	2.8	0	1.6	71
4	13.4	145.0	6.9	0	0	41
5	7.4	38.0	42.8	0	0	93
6	10.8	29.0	98.4	0.4	1	90
7	8.8	48.0	35.0	57.0	26	33
8	11.1	20.0	21.0	NA	0	99
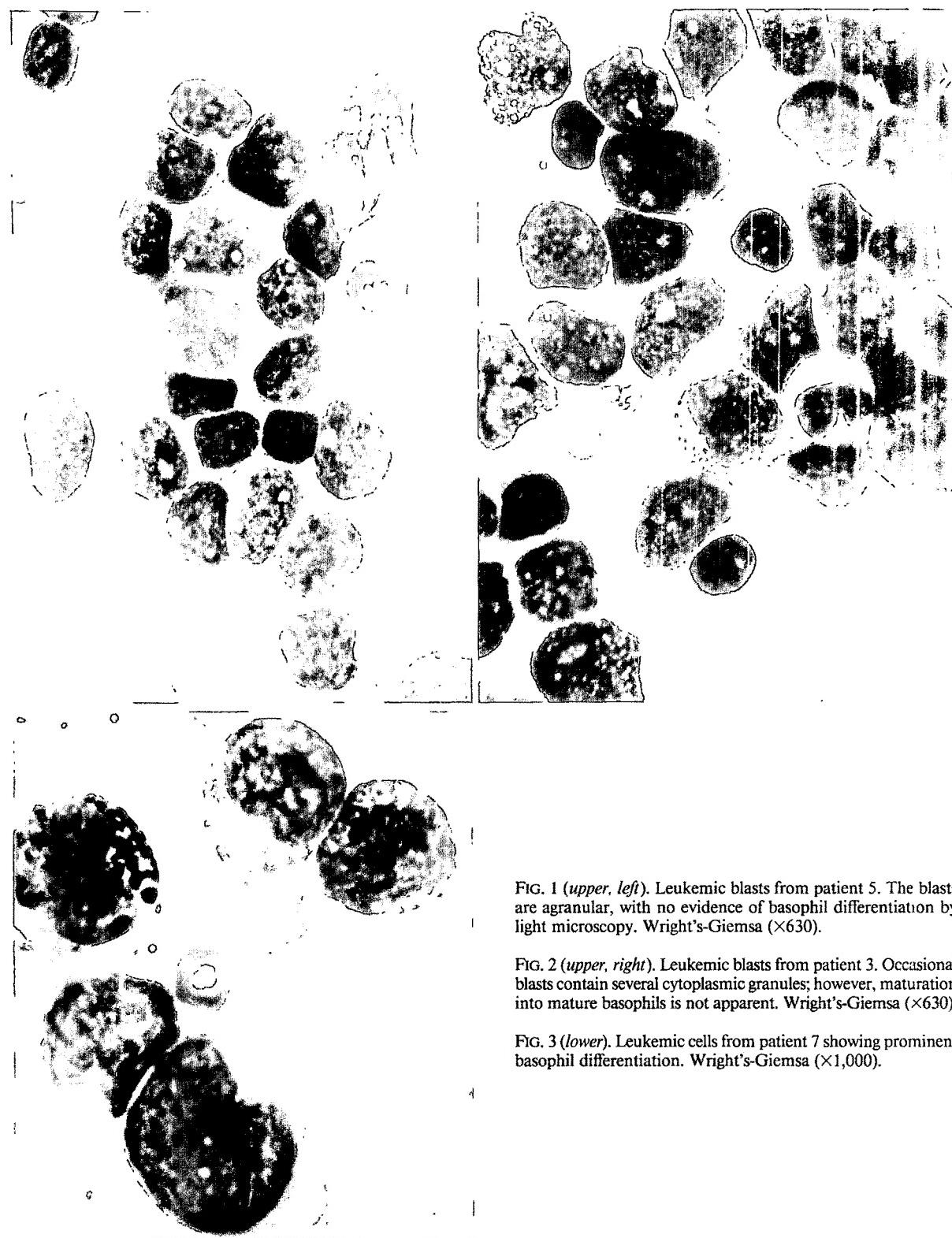
FIG. 1 (*upper, left*). Leukemic blasts from patient 5. The blasts are agranular, with no evidence of basophil differentiation by light microscopy. Wright's-Giemsa (×630).

FIG. 2 (*upper, right*). Leukemic blasts from patient 3. Occasional blasts contain several cytoplasmic granules; however, maturation into mature basophils is not apparent. Wright's-Giemsa (×630).

FIG. 3 (*lower*). Leukemic cells from patient 7 showing prominent basophil differentiation. Wright's-Giemsa (×1,000).

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TABLE 3. CYTOCHEMICAL AND TdT CHARACTERISTICS OF THE BLASTS IN EIGHT CASES
OF ACUTE BASOPHILIC LEUKEMIA

Case No.	Myeloperoxidase (% positive)	Sudan Black B (% positive)	PAS (% positive)	Toluidine Blue	TdT (% positive)
1	Pos‡ (% NA)	Pos (90%)	Neg	Neg	Not done
2	Pos (38%)	Pos (64%)	Neg	Neg	Neg
3	Neg	Neg	Neg	Neg	Neg
4	Neg	Neg	Pos (47%)	Equivocal	Neg
5	Neg	Neg	Neg	Neg	Pos (90%)
6	Neg	Not done	Not done	Not done	Neg
7	Neg	Neg	Neg	Equivocal	Pos (10%)
8	Neg	Neg	Pos (% NA)	Not done	Pos (80%)

PAS = periodic-acid Schiff; TdT = terminal deoxynucleotidyl transferase; Pos = positive; NA = not available; Neg = negative.

cluding blasts) were increased prominently in the bone marrow and blood (Table 2).

Mild morphologic abnormalities involving the neutrophils and mild eosinophilia were identified in one case (case 1). Erythropoiesis and megakaryocytopoiesis, although decreased, appeared morphologically normal in all patients.

In all cases, the sections were significantly hypercellular and diffusely infiltrated by blasts. Basophils were increased in number in the sections from the patients who also had aspirates with increased numbers of basophils. Reticulin stains performed on biopsy specimens from five patients (cases 2, 3, 4, 5, and 7) showed a moderate diffuse increase in fibrosis in two patients (cases 4 and 7).

Cytochemistry and TdT Results

Table 3 shows the results of cytochemical and TdT immunofluorescence studies. Staining for myeloperoxidase was performed in all eight cases. In six, the blasts were negative; in two, they were positive. Similar results were found in the seven cases tested with Sudan Black B. The blasts were negative for periodic acid-Schiff in five cases and positive in two cases. The blasts were negative or equivocal in six cases tested with toluidine blue. When mature basophils were present, the granules were distinctly metachromatic. In the two cases tested (cases 3 and 4), the blasts had negative results for chloroacetate esterase. Seven cases were tested for TdT. In four, the blasts were negative; in the remaining three, they were positive, with an interpretable fluorescent signal seen in 10–90% of the blasts.

Immunophenotypic Analysis

The results of the immunophenotypic analysis are shown in Table 4. In three patients (cases 4, 5, and 7), the immunophenotype of the leukemic cells was consistent with myeloid differentiation. In one patient (case 8), the initial immunophenotypic analysis was non-T, non-B, common acute lymphoblastic leukemia antigen posi-

tive; the blasts were also TdT positive. The blasts from this case contained the t(9;22) chromosomal abnormality. On the basis of the immunophenotypic evidence of a lymphoid process and the ultrastructural evidence of a myeloid component, this case could be viewed as biphenotypic.

Cytogenetic Findings

The cytogenetic findings are summarized in Table 5. Twenty or more metaphase cells were obtained in seven of the eight cases. Two patients were karyotypically normal. One patient had an acquired trisomy 21 clone, and one patient had a deletion of the distal long arm of chromosome 7. The remaining three patients had a Philadelphia chromosome arising from the common 9;22 translocation. Two of the three patients with a Philadelphia

TABLE 4. RESULTS OF IMMUNOPHENOTYPIC ANALYSIS OF
FOUR CASES OF ACUTE BASOPHILIC LEUKEMIA

Antigen	Case 4	Case 5	Case 7	Case 8
CD1	—*	ND	ND	ND
CD2	ND	ND	ND	—
CD3	—	ND	ND	ND
CD4	—	ND	ND	ND
CD5	ND	ND	—	ND
CD7	+†	ND	+	ND
CD8	—	ND	ND	ND
CD9	+	—	+	+
CD10	+	+	ND	+
CD11b	ND	—	ND	ND
CD13	ND	ND	—	ND
CD14	ND	—	—	ND
CD15	ND	—	ND	ND
CD19	+	ND	ND	ND
CD24	—	ND	—	+
CD33	+	+	+	ND
CD34	ND	+	ND	ND
CD61	ND	—	ND	ND
HLA & DR	+	ND	+	+

ND = not done.
* Negative for the antibody tested.
† Positive for the antibody tested.

Acute Basophilic Leukemia

TABLE 5. CYTOGENETIC FINDINGS IN EIGHT CASES OF ACUTE BASOPHILIC LEUKEMIA

Case No.	Speciment Type	Number of Metaphases Analyzed			Karyotype
		Total	Normal	Abnormal	
1	Marrow	No analyzable material			
2	Marrow	20	18	2	46,XY/47,XY,+21
	Blood	10	8	2	46,XY/47,XY,+21
3	Marrow	20	20	0	46,XY
4	Marrow	20	20	0	46,XY
5	Marrow	36	22	14	46,XY/46,XY,del(7)(q32)
6	Marrow	20	0	20	47,XY,+8,t(9;22)(q34;q11)
7	Blood	26	3	23	3 = constitutional: 46,XX, inv(9)(p11q13) 11 = clone 1: 46,XX, inv (9)(p11q13),t(9;22)(q34;q11) 12 = clone 2: 46,XX,inv(9)(p11q13),t(9;22)(q13;q11)
8	Marrow	20	14	6	14 = 46,XX 3 = clone 1: 46,XX,+X,−9,−9,−22,del11(q13),+der(9)t(9;22)(q34;q11),+mar 2 = clone 2: 47,XX,+X,−9,del(11)(q13),t(9;22)(q34;q11),+der(22)t(9;22)(q34;q11) 1 = nonclonal: 49,XX,+X,+17,t(9;22)(q34;q11),+der(22)t(9;22)(q34;q11)

chromosome (cases 6 and 8) also had other acquired abnormalities, and one (case 7) had a second different abnormal clone.

Ultrastructural Morphologic Characteristics

In this group of leukemias, the blast cells were extremely heteromorphic with regard to cell size, nuclear configuration, and organelle content. The ultrastructural morphologic characteristics of the blasts varied within and between individual cases. The blast cells often had a lymphoid appearance, with round, indented, or irregular nuclei; moderately condensed chromatin; prominent nucleoli; and a high nuclear cytoplasmic ratio. The cytoplasm contained mitochondria, rare profiles of rough endoplasmic reticulum, a large Golgi area, granules, and (occasionally) large accumulations of glycogen. Other blasts were myeloid in appearance and were characterized by round nuclei, finely dispersed chromatin, and large nucleoli. The cytoplasm was moderate in amount and contained rough endoplasmic reticulum, a prominent Golgi complex, and granules.

In all eight cases, ultrastructural evidence of basophil differentiation was present in the blast cell population. In addition to basophil differentiation, evidence of mast cell differentiation was found in three of the eight cases. The percentage of blasts showing features of basophil and/or mast cell differentiation was variable, ranging from 20% (case 7) to 90% or greater (cases 1, 2, 4, and 5) (Table 6). The blasts exhibited little or no differentiation along other cell lines.

Three types of granules were observed in the blast cells: immature basophil granules, theta granules, and immature mast cell granules. Immature basophil granules (Fig. 4) were present in 1–90% of the blasts in all eight patients. The granules were membrane bound and occasionally

contained pale, speckled material. Often, the granules were electron lucent and appeared to have been extracted. Both the immature and more typical mature basophil granules could be seen in the same cell. Immature basophil granules usually were peroxidase positive in a characteristic speckled pattern (Fig. 4, inset). Both peroxidase-positive and -negative granules were present in the leukemic cells, often adjacent to each other.

In four cases, theta granules were present in less than 5–75% of the blasts. The theta granules, usually grouped in the Golgi area of the cell, were membrane bound, contained pale amorphous material, and were internally bisected by an electron-dense membrane (Fig. 5). Theta granules have been associated with basophil differentiation,[13] but, in this series of patients, they were never the exclusive evidence for basophil differentiation; all cases had immature basophil granules in addition to theta granules. Most often, the blasts contained only theta granules, although rare cells contained theta granules and immature basophil granules. Theta granules usually were peroxidase negative, although in rare granules the bisecting

TABLE 6. ULTRASTRUCTURAL FEATURES OF THE BLASTS IN EIGHT CASES OF ACUTE BASOPHILIC LEUKEMIA

Case No.	Immature Basophil Granules*	Theta Granules*	Immature Mast Cell Granules*
1	15%	<5%	75%
2	15%	75%	Absent
3	1%	75%	Absent
4	90%	<5%	Absent
5	90%	Absent	Absent
6	10%	Absent	30%
7	20%	Absent	Absent
8	10%	Absent	40%

* Percentage of blasts with this morphologic feature.

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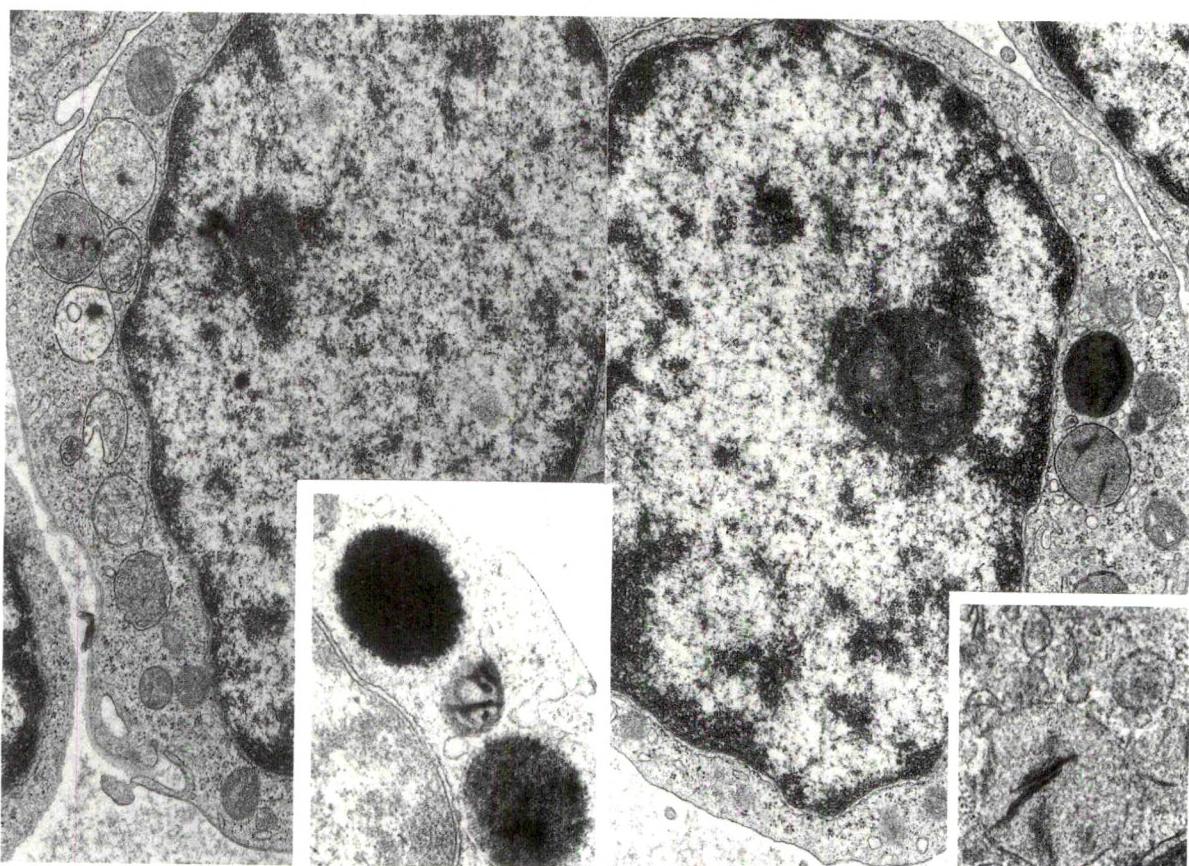
FIG. 4 (*left*). Bone marrow blast from patient 4 containing several large cytoplasmic granules. The granule contents vary from pale and flocculent to moderately electron-dense, with a central electron-density. Uranyl acetate, lead citrate (×14,000). *Inset.* Higher magnification micrograph of two peroxidase-positive granules. Lead citrate (×28,000).

FIG. 5 (*right*). Blast from the blood of patient 2 illustrating theta-type granules. Uranyl acetate, lead citrate (×15,000). *Inset.* A theta granule from the bone marrow of patient 3 shows electron-dense peroxidase reaction product along the internal bisecting membrane. Lead citrate (×41,000).

membrane was positive (Fig. 5, inset); some cells contained peroxidase-negative theta granules intermixed with peroxidase-positive granules.

In three cases, immature mast cell granules were present in 30–75% of the blasts. Usually, the blasts contained basophil or mast cell granules, although in one case (case 6) rare blasts with both types of granules were observed (Fig. 6). The immature mast cell granules were located in the Golgi area and often were encircled by profiles of rough endoplasmic reticulum. Granule contents were characterized by primitive scrolls and lamellae surrounded by pale amorphous material (Fig. 6, inset). Ultrastructural localization of peroxidase showed that the mast cell granules were uniformly peroxidase negative, whereas the Golgi apparatus rarely contained small peroxidase-positive vesicles.

In addition to basophil/mast cell blast differentiation, maturation of leukemic cells to more mature and fully mature basophils and mast cells occurred in four cases

(cases 1, 3, 4, and 7). The more mature mast cell precursors were spindle-shaped cells with irregular nuclei and multiple, small nucleoli. The abundant cytoplasm was elongated, and mast cell granules usually were located at one pole of the cell. The granule content was moderately electron dense and contained well-developed scrolls and lamellae. Immature basophils were characterized by round to lobulated nuclei with condensed chromatin and small nucleoli. The cytoplasm contained immature and mature basophil granules.

Lymph Node Biopsy Specimen

The cervical lymph node biopsy specimen from one patient (case 4) showed near-total effacement, with rare residual lymphoid follicles. The node was diffusely infiltrated by leukemic blasts (Fig. 7A). The blasts were small, with round, oval, or slightly irregular nuclei. The chromatin was finely dispersed, and frequent single prominent
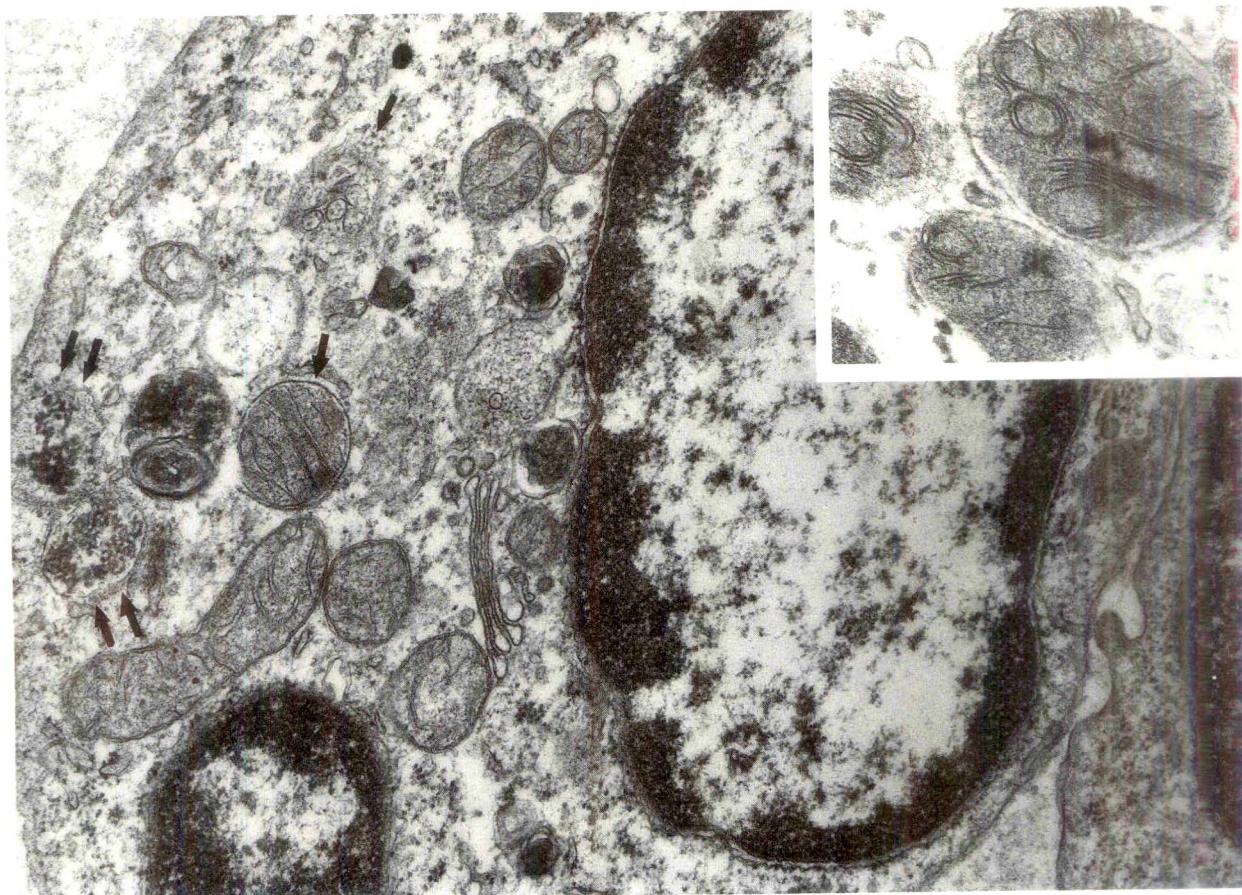
FIG. 6. Blast from the blood of patient 6 contains both basophil granules (*double arrows*) and mast cell granules (*arrow*) in the cytoplasm. Uranyl acetate, lead citrate (×34,000). *Inset.* High magnification of a mast cell granule from the same patient showing the characteristic scrolls and lamellae. Uranyl acetate, lead citrate (×64,000).

nucleoli were evident. Mitoses were numerous. Immature eosinophils were not apparent. The blasts were negative for chloroacetate esterase, toluidine blue, lysozyme, alpha-1-antichymotrypsin, and Leu-M1.

Ultrastructural studies revealed diffuse infiltration by blasts, with no evidence of cell junctions. The nuclei were round to deeply indented and contained prominent, single nucleoli and finely dispersed chromatin. The cytoplasm was moderate in amount and contained abundant polyribosomes, rare linear profiles of rough endoplasmic reticulum, numerous mitochondria, and a prominent Golgi zone. Approximately 10% of the blasts contained large immature basophil granules, usually located in the Golgi area of the cytoplasm (Fig. 7*B*). The granule contents varied from pale to moderately electron dense.

DISCUSSION

In this report, we describe eight patients with acute basophilic leukemia. The cases were selected from 455 cases of acute leukemia that were examined by electron mi-

croscopy. Cases exhibiting ultrastructural evidence of basophil differentiation of the blasts were classified as acute basophilic leukemia.

In two of the eight cases, prominent basophil differentiation of the blasts was evident by light microscopic examination, and basophils were increased in both the blood and marrow. In the other six cases, basophils were rare or absent; basophilic involvement was not apparent until ultrastructural features were examined. In some of these latter six cases, rare to occasional blasts contained a few granules that could have represented basophil granules, but maturation into more mature basophils was not apparent or was minimal. When stained with toluidine blue, the granules were negative or equivocal for metachromasia. Because granules in the mature basophils were metachromatic, the lack of positivity in the blasts may have resulted from the immaturity of the granules.

On the basis of light microscopic findings, four of the six cases without prominent basophilia were initially classified as acute myeloid leukemia (French–American–British classification M1). In one case, this diagnosis was

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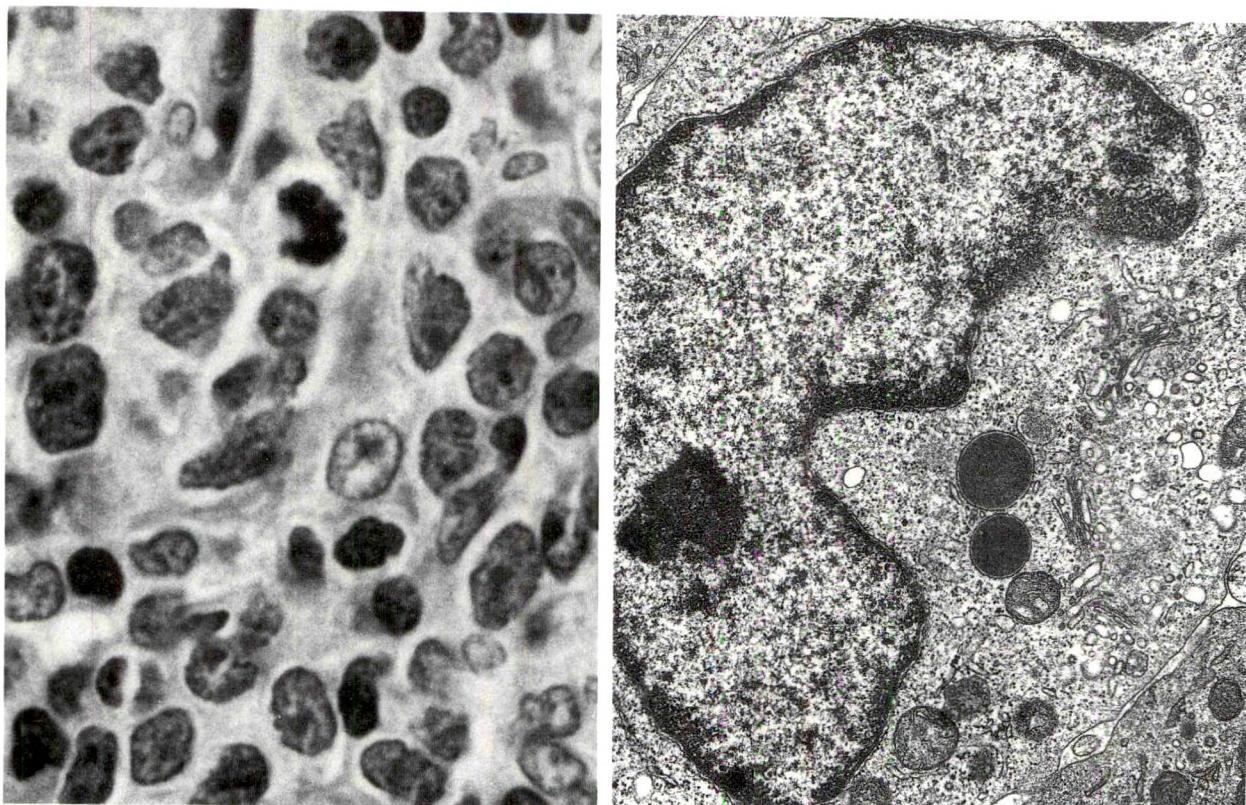
FIG. 7 (*left*). Primitive leukemia blasts infiltrating lymph node from patient 4. Hematoxylin and eosin (×630). (*Right*) Electron micrograph of a blast cell from the lymph node of patient 4. The golgi area of the cell contains two large, moderately electron-dense immature basophil granules. Uranyl acetate, lead citrate (×16,000).

supported by the myeloperoxidase positivity of the blasts and in the other three, by immunophenotypic analysis. One of the six cases had immunophenotypic characteristics that were typical of acute lymphoblastic leukemia, and initially this case was diagnosed as such. In view of the ultrastructural findings, we interpret this case as biphenotypic acute leukemia. By light microscopic and cytochemical analysis, two of the six cases appeared to be undifferentiated; immunophenotypic analysis was not performed.

In all eight cases, ultrastructural analysis revealed immature basophil granules and provided evidence of basophilic differentiation of the blasts. Theta granules, which have been associated with early basophil differentiation,[13] also were present in blasts from four patients. In three cases, ultrastructural analysis provided evidence of mast cell differentiation, with rare individual cells showing basophil and mast cell features.

No clinical features distinguished this group of patients from others with acute myeloid leukemia. None of the patients showed any clinical signs of excess histamine release, a feature previously reported by Wick and

associates[4] in three of four cases of acute myeloid leukemia with basophil differentiation.

Few other cases of well-documented *de novo* acute basophilic leukemia have been reported. Wick and associates[4] reported four adult patients with "acute non-lymphocytic leukemia with basophilic differentiation." Those cases were diagnosed primarily on the basis of light microscopic examination and appear similar to our cases. More than 10% of the blasts were granular, and the blasts were myeloperoxidase negative but were metachromatic with toluidine blue. Ultrastructural analysis performed on the blasts from one of their patients revealed basophil differentiation.[4] Kurosawa and associates[18] also reported a case of congenital acute basophilic leukemia in which the blasts, on light microscopic examination, contained large granules that were shown to be basophil granules on ultrastructural analysis. In addition, Lawlor and associates[19] described a patient with acute lymphoblastic leukemia, similar to one of our patients (case 8), in whom the blasts had ultrastructural features of basophil/mast cell granules.

Most reported cases of basophilic leukemia appear to

be Philadelphia chromosome–positive chronic myeloid leukemia in accelerated phase, with increased mature and immature basophils.[20–25] Also described in the literature are several cases of blast crisis of chronic myeloid leukemia[10,13,26–29] or (rarely) other prior myeloproliferative disorders,[30] with ultrastructural evidence of basophil differentiation of the blasts. Several patients presenting with Philadelphia chromosome–positive acute leukemia or acute undifferentiated leukemia with basophil or mast cell differentiation of the blasts have been reported as well.[10,13,27] The cases previously reported from this laboratory[10,27] were not included in the current study because they exhibited prominent dysplasia of other myeloid cell lines in addition to basophils.

Cytogenetically, our cases of acute basophilic leukemia were heterogeneous. Three of the seven patients studied had a Philadelphia chromosome arising from the common 9;22 translocation. No patient had a history of chronic myeloid leukemia. Two patients were karyotypically normal. No patient had a 6;9 translocation[2] or 12p abnormality,[3] cytogenetic abnormalities that have been associated with acute myeloid leukemia and basophilia.

Although most data support the view that basophils and mast cells originate from the bone marrow, it is not clear whether they share a common precursor cell.[31] Ultrastructural analysis in our patients with acute basophilic leukemia revealed that some cases had blasts containing basophil granules, with other cells (in the same case) containing mast cell granules; rarely, cells with both types of granules were present. Similar ultrastructural findings have been reported in cases of chronic myeloid leukemia,[31] blast crisis of chronic myeloid leukemia,[10,13,27,28] Philadelphia chromosome–positive acute leukemia,[10,27] and other myeloproliferative disease.[31] Taken as a whole, these ultrastructural observations provide evidence for a common origin of mast cells and basophils, as proposed by Zucker-Franklin.[31]

In summary, we report eight cases of acute basophilic leukemia. These cases represent less than 2% of the cases of acute leukemia studied ultrastructurally during the period required to identify the eight cases. Because basophil involvement was not apparent by light microscopic examination in many of the cases, ultrastructural analysis was required for diagnosis. The cytogenetic findings in these cases were diverse; three of the eight cases were associated with the Philadelphia chromosome. None of the cases had other chromosome abnormalities previously associated with increased basophils. The diagnosis of acute basophilic leukemia can be elusive but may be considered when there is light microscopic evidence of differentiation into basophils; when the blasts contain granules that resemble basophil granules by light microscopic examina-

tion; when the leukemia appears undifferentiated; or when the acute leukemia is positive for the Philadelphia chromosome.

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Original Article

Fulminant Childhood Hemophagocytic Syndrome Mimicking Histiocytic Medullary Reticulosis

An Atypical Form of Epstein-Barr Virus Infection

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Ten cases of pediatric fulminant hemophagocytic syndrome, encountered between 1986 and 1989, are described. They occurred in the summer, and the patients presented with fever, jaundice, hepatosplenomegaly, pancytopenia, coagulopathy, and abnormal liver function. Bone marrow studies revealed infiltration by atypical T-lymphoid cells, rare B immunoblasts, and mature histiocytes with hemophagocytosis. Initially, histiocytic medullary reticulosis was suspected in six cases. The clinical course was characterized by rapid deterioration, with a mean period of 16 days from onset of fever to death. The main causes of death were coagulopathy with multiple organ failure and opportunistic infection. In seven of eight cases studied by serologic assay and Southern blot hybridization, acute or active Epstein-Barr virus (EBV) infection was documented. It is suggested that an atypical or fulminant form of primary EBV infection distinct from classic infectious mononucleosis was prevalent in previously healthy children in Taiwan. Younger age involvement and seasonal clustering were characteristic of the disorder described. (Key words: Hemophagocytic syndrome; Histiocytic medullary reticulosis; Epstein-Barr virus; Infectious mononucleosis) Am J Clin Pathol 1991;96:171–176

Hemophagocytic syndrome is a heterogeneous disease with disordered macrophage activation that may be related to subtle genetic or immunologic defects.[1] Some cases have been designated as familial erythrophagocytic lymphohistiocytosis, a lesion that is characterized by autosomal recessive inheritance and early disease onset.[2,3] Other cases represent virus- or infection-associated hemophagocytic syndromes (VAHS or IAHS) related to Epstein-Barr virus (EBV), cytomegalovirus (CMV), adenovirus, salmonella, tuberculosis, or other infectious agents.[4-7] Most VAHS-affected patients have acquired immunosuppression[7] or an established immune deficiency syndrome such as Chédiak-Higashi syndrome[8] or X-linked lymphoproliferative syndrome.[9] Demonstration of atypical lymphoid or immunoblast-like cell in the bone marrow and lymphoid organs in some cases suggests that histiocytic medullary reticulosis (HMR) may be present.[10]

The prevalence and seasonal clustering of an acute fulminant form of hemophagocytic syndrome has been reported in Taiwan, occurring especially in toddlers.[10,11] Ten cases were encountered from 1986 to 1989 in the Department of Pediatrics, National Taiwan University Hospital. The patients' clinical, laboratory, pathologic, and virologic features were analyzed.

MATERIALS AND METHODS

Patients

From 1986 to 1989, 12 patients presented with a fulminant septic-like process and a hemophagocytic syndrome. Later, two cases were shown to result from *Salmonella typhi* or *Pseudomonas aeruginosa* sepsis; each afflicted patient recovered within two weeks after anti-

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biotic treatment. The remaining ten cases showed no apparent underlying disorders and were investigated retrospectively for a possible association with EBV and CMV. The patients' clinical histories, presenting symptoms and signs, and hematologic and other laboratory data were reviewed.

Hematologic and Pathologic Studies

The peripheral blood smears and bone marrow aspirates from the ten patients were stained with a modified Giemsa (Liu) stain. Ten bone marrow core biopsies, four liver biopsies, and three autopsies were performed. The tissues from bone marrow and other lymphoid organs were fixed in B5 solution or neutral buffered formalin, processed in a routine fashion, and stained with hematoxylin and eosin. To investigate the infiltrating lymphohistiocytic cells in the tissues, immunoperoxidase studies were performed on paraffin sections from all ten patients, and fresh-frozen specimens from two patients (cases 2 and 4), with a panel of monoclonal antibodies and antisera specific for T and B lymphocytes and histiocytes as previously described.[10]

Virologic Studies

Serologic Studies. Serum was available from six patients (cases 3, 5, 6–8, and 10) for viral serologic studies. The EBV-specific antibody titers to viral capsid antigens were determined by an indirect immunofluorescence technique (Gull Laboratories, Inc, Salt Lake City, UT). The antibodies to EBV-nuclear antigens were determined by an anticomplement immunofluorescence test (Organon Teknika, Holland); CMV IgM antibody was determined by an enzyme-linked immunosorbent assay method (Behring, Holland); and the IgG antibody to CMV was determined by a complement fixation method (Whittaker M.A. Bioproduct, MD).[12-14]

Southern Blot DNA Hybridization Studies. Fresh-frozen tissues from two patients (cases 2 and 4) were available for Southern blot studies to investigate a possible association between their disease and EBV or CMV infection.[10,15] For the Southern blot study of EBV, DNA was extracted from frozen lymph node specimens. Fifteen micrograms of DNA was digested with the BamH1 restriction endonuclease, loaded onto 0.8% agarose gel, electrophoresed, and blotted with a Zeta-probe (Biorad, Richmond, CA) nylon membrane. The baked membrane then was prehybridized and hybridized for EBV with the use of probes of EBV BamH1-A and W fragments at a high radioactivity of 5×10^8 counts per minute (cpm)/μg. For the study of CMV, the same nylon membrane for EBV was washed to strip off the preexisting radioactive substance and then rehybridized for a CMV probe (EcoRI-digested, 27.5-kb DNA fragment, kindly provided by Dr. Eng-Shang Huang).[16]

RESULTS

Clinical Characteristics

Demographic Features. Nine of ten patients were infants or toddlers, with a median age of 18 months. There were six boys and four girls. Eight of ten cases were from urban areas. Eight of the cases developed between May and July.

Family and Histories. Patient 9 had a history of chronic diarrhea in early infancy, with a body weight in the third percentile at presentation. None of the remaining patients had a positive family history or personal history suggesting an immune deficiency syndrome.

Clinical Manifestations. All patients had a febrile prodrome ranging from one to four weeks associated with frequent upper respiratory tract symptoms. Subsequently, full-blown manifestations of jaundice (nine of ten), hepatomegaly (ten of ten, mean 5.8 cm below right costal margin), splenomegaly (nine of ten, mean 2.3 cm below left costal margin), pancytopenia (ten of ten), coagulopathy (eight of eight), and abnormal liver function (ten of ten) developed, with hepatomegaly consistently more prominent than splenomegaly in all instances. Pulmonary and pleural infiltrates were noted in four cases. Ascites was noted in eight cases. Lymphadenopathy either was not evident or consisted of pea-size palpable nodes in all patients.

Laboratory Findings. Progressive neutropenia, lymphopenia, thrombocytopenia, and hyporegenerative anemia developed in all of the patients within days of presentation. Conjugated hyperbilirubinemia with elevation of levels of hepatic enzymes (aspartate/alanine aminotransferase) and biliary enzyme (alkaline phosphatase) denoted extensive liver parenchymal damage. The albumin and globulin concentrations were decreased. Serum ammonia levels were normal in six patients tested. The prothrombin time and partial thromboplastin time were prolonged in the eight patients tested. Among these, two patients (patients 1 and 9) had evidence of a consumption coagulopathy (decreased fibrinogen, increased fibrin degradation product, and positive fibrin monomer complexes detected by the plasma protamine paracoagulation test).[17] Serum triglyceride levels were elevated in seven patients, and total cholesterol levels were elevated in one of nine patients tested (Table 1). Blood urea nitrogen and creatinine levels were within normal limits, except in patient 10, in whom azotemia later developed after prolonged and complicated secondary infections. Cerebrospinal fluid examination in cases 1, 3, and 4 showed xanthochromia and mild lymphocytosis. Bacteria cultures drawn at presentation from blood or other tissue fluid (urine, cerebrospinal fluid, ascites, and pleural effusion) were negative,

Hemophagocytic Syndrome

TABLE 1. LABORATORY DATA AT PRESENTATION

Cases	1	2	3	4	5	6	7	8	9	10
WBC	2.20	1.70	2.00	18.20	1.60	1.10	2.00	1.00	3.20	13.90*
Hb	101	73	88	114	106	86	71	68	96	92*
Plt	42	113	39	33	36	8	22	27	14	106*
Alb	41	28	22	20	23	41	33	24	23	ND
Glo	27	20	14	16	14	22	18	18	19	ND
AST	9.45 (567)	4.47 (268)	5.68 (341)	3.47 (208)	2.28 (137)	2.92 (175)	5.80 (348)	9.35 (561)	40.34 (2,420)	62.85 (3,770)
ALT	1.23 (74)	2.22 (133)	3.73 (224)	1.28 (77)	1.18 (71)	1.22 (73)	1.50 (90)	3.12 (187)	6.75 (405)	9.67 (580)
Bil (T)	159 (9.3)	147 (8.6)	135 (7.9)	17 (1.0)	139 (8.1)	200 (11.7)	9 (0.5)	77 (4.5)	181 (10.6)	147 (8.6)
Bil (D)	77 (4.5)	92 (5.4)	94 (5.5)	5 (0.3)	82 (4.8)	139 (8.1)	5 (0.3)	63 (3.7)	128 (7.5)	109 (6.4)
ALP	3.3 (198)	10.5 (628)	14.5 (867)	2.2 (134)	32.7 (1,963)	ND	8.0 (482)	56.7 (3,401)	8.2 (492)	20.9 (1,253)
LDH	60.8 (3,650)	ND	115.5 (6,930)	31.8 (1,906)	38.6 (2,315)	ND	82.3 (4,940)	126.5 (7,590)	86.2 (5,170)	386.6 (23,190)
TG	7.70 (682)	1.12 (99)	7.76 (687)	0.07 (6)	5.49 (486)	ND	2.18 (193)	5.17 (458)	2.01 (178)	7.26 (643)
CHO	2.66 (103)	2.64 (102)	2.43 (94)	0.52 (20)	4.91 (190)	ND	2.12 (82)	11.84 (458)	2.53 (98)	3.21 (124)

Abbreviations and units: WBC = white blood cell count, 10^9/L; Hb = hemoglobin, g/L; Plt = platelet count, 10^9/L; Alb = albumin, g/L; Glo = globulin, g/L; AST = aspartate aminotransferase, μmol/L (IU/L); ALT = alanine aminotransferase, μmol/L (IU/L); Bil (T/D) = bilirubin (total/conjugated), μmol/L (g/dL); ALP = alkaline phosphatase, μkat/L (IU/L); LDH = lactate dehydrogenase, μkat/L (IU/L); TG = triglycerides, mmol/L (mg/dL); CHO = cholesterol, mmol/L (mg/dL).

Normal range in this laboratory: Alb = 37–52 g/L; Glo = 22–31 g/L; AST = 0.08–0.52 μkat/L (5–31 IU/L); ALT = 0–0.52 μkat/L (0–31 IU/L); Bil (T/D) = 3–17/0–7 μmol/L (0.2–1.0/0–0.4 mg/dL); ALP = 1.1–4.0 μkat/L (64–238 IU/L); LDH = 0.30–7.67 μkat/L (18–460 IU/L); TG = 0.56–1.47 mmol/L (50–130 mg/dL); CHO = 3.36–6.47 mmol/L (130–250 mg/dL).

* Initial data from referring hospital.

ND = no data.

except in patient 4, whose blood culture yielded *Escherichia coli* on admission. Patients 1, 2, 4, 8, and 10 had documented secondary infection in terminal or autopsy specimens with Aspergillus species, *Candida albicans, Bacteroides fragilis, P. aeruginosa,* and *C. albicans,* respectively.

Treatment and Outcome. Blood components and antibiotics were administered aggressively in all patients. In general, no specific therapy was given because of the patients' rapidly deteriorating conditions or because, initially, the condition was not diagnosed correctly. The exceptions were the use of acyclovir in case 5 and steroids in cases 2, 5, and 7. None of the patients survived. The main causes of death were coagulopathy with multiple organ failure and secondary infection. The duration from disease onset to death ranged from 1 to 4 weeks (mean, 11 days) and from 1 to 10 days after full-blown clinical manifestations appeared, when patient 10 (the only patient who was older than four years of age and survived for 55 days after fever onset) was excluded.

Hematologic and Pathologic Features

Peripheral Blood. Smears revealed cytopenia without left shifting, except in case 9, for which normoblasts and metamyelocytes were present on the peripheral blood smears. Few (0.00–0.04) atypical lymphocytes were present in peripheral blood smears, except in cases 8 and 10, in which atypical lymphocytosis (0.10 and 0.15) was noted in the initial blood smear. Blasts were absent.

Bone Marrow. Hemophagocytic histiocytosis (HPH) accompanied by varying degrees of infiltration of lymphoid cells, including immunoblasts and plasma cells, was found in all ten specimens (Fig. 1). Cases 2–7 showed infiltration of bone marrow or other organs by atypical histiocyte-like cells. These were designated as HMR on morphologic and histochemical grounds.[18] With immunoperoxidase and immunophenotypic studies, the immunoblasts or atypical histiocyte-like cells were of polyclonal B lineage in case 4 and T lineage in the other nine cases. Variable depletion of myeloid, erythroid, and megakaryocytic series was observed in the marrow samples. In autopsy specimens, areas of necrosis were noted in varying degrees (Fig. 2).

Liver. Cases 1, 2, 4, 7, and 10 had adequate liver specimens for study. HPH and atypical lymphoid cells were found in the portal areas and sinusoids. There was a variable degree of fatty metamorphosis of hepatocytes. A variable amount of patchy coagulation necrosis was observed, present mainly in the peripheral portion of liver parenchyma and the periportal areas. Eosinophilic bodies (possibly representing degenerating phagocytic histiocytes) could be found in liver parenchyma and sinusoids (Fig. 3).
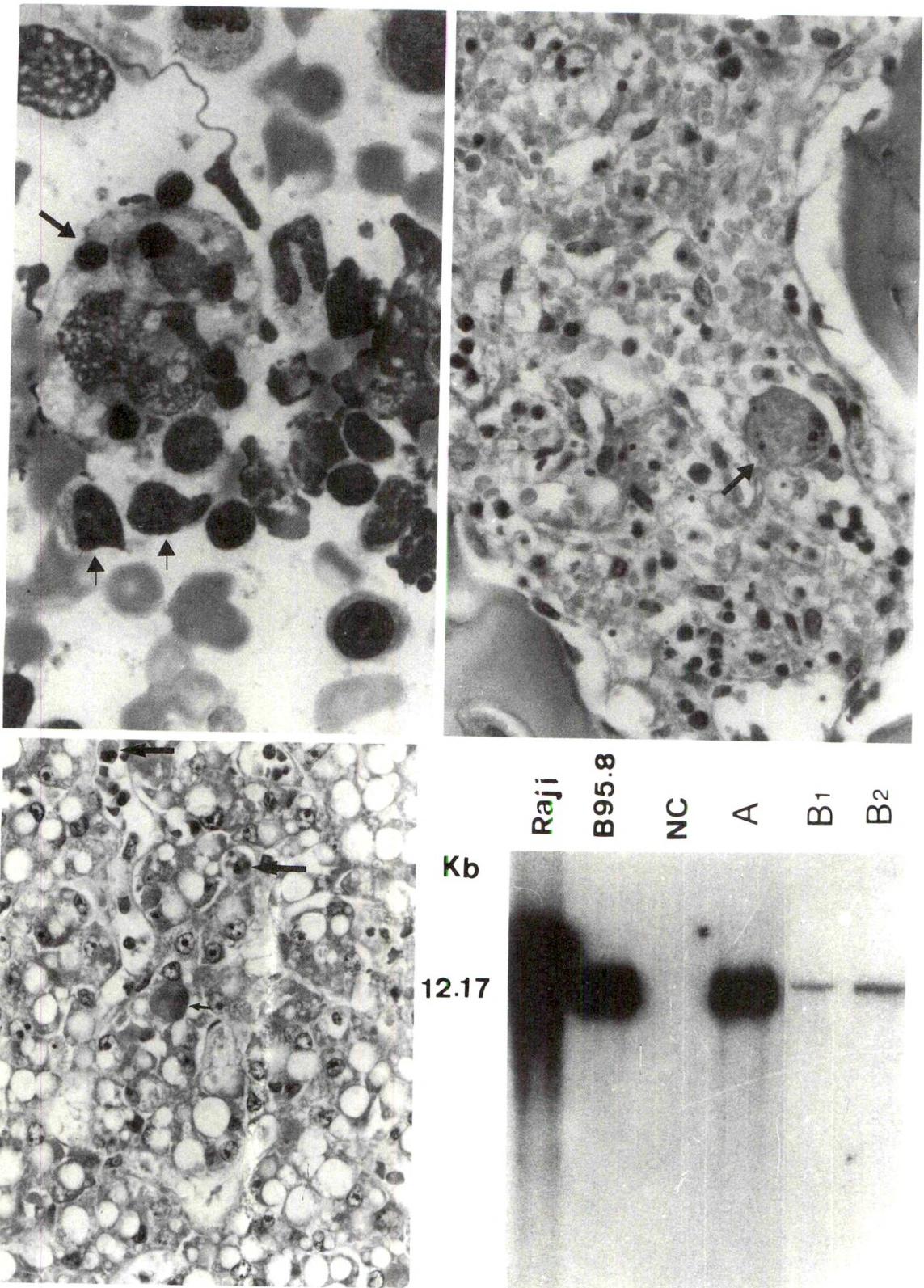
FIG. 1 (*upper, left*). Bone marrow aspiration smear at early stage shows prominent histiocytic hemophagocytosis (*long arrow*) and atypical histiocyte-like cell or immunoblast (*short arrows*) infiltration.

FIG. 2 (*upper, right*). Bone marrow at terminal stage shows marked cellular depletion and massive necrosis, but histiocytic hemophagocytosis (*arrow*) is present.

FIG. 3 (*lower, left*). Liver histology shows fatty metamorphosis of hepatocytes. Hemophagocytic histiocytes (*long arrows*) and eosinophilic bodies (*short arrow*) are found in the sinusoids.

FIG. 4 (*lower, right*). Southern blot hybridization with EBV-BamH1-A fragment in cases 2 (line A, lymph node) and 4 (line B1, lymph node; line B2, spleen) shows a clear hybridization band at 10-kb locations corresponding to EBV-A fragment shown in Raji and B95.8 cell lines.

Autopsy. Three autopsies (cases 1, 2, and 4) were performed. In case 4, there was diffuse infiltration of lymph nodes by atypical lymphoid cells or immunoblasts that resembled a large cell/immunoblastic lymphoma. In cases 1 and 2, lymphoid depletion with extensive hemophagocytosis and necrosis was found. In all three patients, the same infiltrate was found in the spleen, lungs, and thymus and was relatively sparse in nearly all of the visceral organs, except the brain, wherein no atypical cells were found.

Virologic Studies

Serologic Studies. Cases 7, 8, and 10 had a documented acute EBV infection. Cases 3 and 6 were in a window stage in which viral capsid antigen (VCA)-IgM antibodies were no longer detectable and EBV nuclear antigen (EBNA) antibodies were not yet detectable.[12] In summary, five of six cases with viral serologic studies had evidence of acute EBV infection. There was no evidence of acute CMV infection in any case analyzed (Table 2).

Southern Blot Hybridization Studies. Southern blot hybridization using the EBV BamHI-A fragment radioactive probe showed a clear hybridization band at 10 Kb, corresponding to the molecular size of the EBV-A fragment in two patients (patients 2 and 4) (Fig. 4). The intensity of the hybridization band for case 4 was comparable to that of Raji and B95.8 lines, representing 50–100 copies of EBV genomes per cell. The intensity of the hybridization band was much weaker for case 2, suggesting a lower percentage of EBV-containing cells in tissues.

DISCUSSION

A fatal histiocytic hemophagocytic syndrome has been encountered that mainly affects previously healthy infants or toddlers. The clinical differential diagnosis included a septic process, VAHS or HMR. The presence of atypical large cells and hemophagocytosis led to the suspicion of

HMR in some cases. However, case-clustering in the summer season suggested an infectious process. Application of serologic, Southern blot, and *in situ* DNA hybridization studies indicated a strong association with acute or active EBV infection. However, the absence of peripheral lymphadenopathy, and the profound liver involvement, paucity of peripheral atypical lymphocytosis, severe coagulopathy, and rapidly progressive bone marrow failure distinguished this process from the usual manifestations of classic infectious mononucleosis. These patients, who might have had subtle immunologic defects related to age or a genetic abnormality, apparently had an atypical or fulminant form of infectious mononucleosis.

One important aspect of these cases was their frequent confusion with HMR. HMR was described first by Scott and Robb-Smith[19] and only later was designated as a synonym for malignant histiocytosis.[18] HMR has been reported frequently in particular geographic locations that correspond to EBV-prevalent areas, such as Uganda, mainland China, and Taiwan.[11,20,21] Recently, most of the previously diagnosed cases of HMR have been redesignated as VAHS or T-cell lymphoma.[22,23] The infiltrating atypical large cells in most of the current cases were T cells, raising the possibility that these patients had either VAHS or T-cell lymphoma. However, the possibility of T-cell lymphoma is unlikely because lymphoid depletion was shown in terminal specimens. It was demonstrated that at least some cases of HMR, according to previous criteria, were actually an atypical form of EBV infection. In other words, the clinicopathologic features of the current cases are similar to those described by Wilson as fatal EBV-VAHS.[24] Previously described VAHS frequently occurred in immunosuppressive conditions, and there was no age predilection. In contrast, nine of our ten patients were previously healthy toddlers. There was no family history suggesting a diagnosis of familial erythrophagocytic lymphohistiocytosis, or X-linked lymphoproliferative syndrome in the male patients.

Taiwan is an endemic area for EBV. The association of nasopharyngeal carcinoma and peripheral T-cell lymphoma with EBV has been demonstrated.[25,26] The clustering occurrence and young age involvement accompanied by the findings of serologic and DNA hybridization studies demonstrated that this fatal VAHS occurring in toddlers might be an atypical form of primary EBV infection. Most people in Taiwan contract EBV infection at a young age but have no symptoms.[27,28] It has been speculated that the intimate relationships reflected in Chinese family culture, such as prechewing food for children, and high infection rates in adults could contribute to early acquisition of EBV infection. The characteristic summer clustering must have important implications in

TABLE 2. SEROLOGIC STUDY OF EBV AND CMV INFECTION

Case No.	EBV-VCA IgG	EBV-VCA IgM	EBNA	CMV IgG	CMV IgM
3	1:40+	1:10−	1:2−	1:2−	1:10−
5	1:160+	1:10−	1:2+	1:2−	1:10−
6	1:160+	1:10−	1:2−	1:2−	1:10−
7	1:10+	1:10+	1:2−	1:8+	1:10−
8	1:40+	1:10+	1:2−	1:2−	1:10−
10	1:160+	1:10+	ND	1:2−	1:10−

ND = no data.

EBV-VCA IgM+, EBV-VCA IgG+, and EBNA denote current primary EBV infection; EBV-VCA IgM, EBV-VCA IgG+, and EBNA denote recent (window stage) infection and are consistent with, although not diagnostic for, primary EBV infection.[12]

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this syndrome because no seasonal clustering has been observed in classic infectious mononucleosis. Additional studies are needed to clarify whether higher infective dosages, different infective strains, interactions with other microorganisms active in summer, or other factors contribute to this syndrome.

These patients had a rapidly fatal course without exception, in contrast to the 40% mortality reported in other VAHS series in which only supportive treatment was provided.[4,7] In other words, the prognosis of the hemophagocytic syndrome is even poorer in these previously well children than in those with acquired immunosuppressive conditions. EBV-VAHS was shown to be a permissive infection of EBV and a disorder of host-uncontrolled histiocyte proliferation.[29,30] Acyclovir, and even an immunosuppressive treatment such as the VP-16 regimen, would be worth trying in an attempt overcome the extremely poor prognosis.[31,32] The clinical course in these patients had two distinct stages: a mild upper respiratory infection–like prodromal stage and a fulminant disseminated multiple-organ-failure stage. This observation is consistent with reports by Mroczek and associates.[9] Our study indicates that an aggressive immunosuppressive protocol should be started as early as possible in the later stage of the disease.

These findings indicate that a fatal form of primary EBV infection mimicking HMR threatens the lives of previously healthy Taiwanese children; summer clustering is characteristic of the disease. The treatment regimen should be more aggressive to improve the patient's prognosis.

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Immunoblastic Lymphoma with Abundant Clear Cytoplasm

A Comparative Study of B- and T-Cell Types

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The morphologic, phenotypic, molecular genetic, and clinical features of 34 cases of clear-cell immunoblastic lymphoma (IBLC) are described. Sixteen cases were of B-cell type (IBLC-B) and 18 cases were of T-cell type (IBLC-T). There were no significant differences in the morphologic characteristics of the neoplastic cells in the two types, although IBLC-B was less likely to be polymorphic than IBLC-T. Interfollicular proliferation, a higher mitotic rate, infiltration by eosinophils, and an increase in capillary-sized blood vessels were also features of IBLC-T, whereas necrosis and fibrosis were more extensive in IBLC-B. Patients with IBLC-B were predominantly female, whereas those with IBLC-T were predominantly male. The mean age was 62 years for those with IBLC-B and 46 years for those with IBLC-T. Patients with IBLC-B usually had lower-stage disease, but there was no significant difference in survival rate between those with IBLC-B and those with IBLC-T. Although most cases of IBLC have been considered to be of peripheral T-cell origin, the authors conclude that IBLC-B is more common than previously considered and that clear-cell morphologic characteristics are not a reliable indicator of T-cell type. (Key words: Immunoblastic lymphoma; Clear cell; B-cell lymphoma; Peripheral T-cell lymphoma; Non-Hodgkin's lymphoma) Am J Clin Pathol 1991;96: 177–183

Non-Hodgkin's lymphoma (NHL) consists of a heterogeneous group of lymphoid neoplasms; the origin of which usually can be determined by phenotypic or molecular genetic analyses. Morphologic features also have been used to predict the cellular origin in several types of NHL.[1,2] In the International Working Formulation,[3] clear-cell immunoblastic lymphoma (IBLC) is characterized by a diffuse proliferation of transformed lymphoid cells with abundant, optically clear cytoplasm. Since the first report of peripheral T-cell lymphoma (PTCL) by Waldron and associates,[4] clear-cell morphology has been considered one of the distinctive features of PTCL.[5–13] However, some authors also have observed this feature in cases of B-cell lymphoma.[7,8,10,12] These preliminary observations led us to review our experience with IBLC of B-cell type (IBLC-B) because, to our knowledge, no detailed studies of this type of NHL have been reported. Herein, we describe the clinicopathologic features of IBLC-B and compare them with those of IBLC of T-cell type (IBLC-T).

MATERIALS AND METHODS

Patients

Thirty-four cases of IBLC in the Nebraska Lymphoma Registry (1982–1989) were studied. The diagnosis of IBLC was based on the histopathologic criteria of the International Working Formulation.[3] Special attention was given to the exclusion of cases in which the cells had an "empty" cytoplasmic appearance resulting either from poor tissue processing or from degeneration in the vicinity of necrotic areas. The following clinical information was recorded in each case at the time of diagnosis: age, sex, date of diagnosis, site of biopsy, stage of disease, type of therapy, and length of survival in months. The clinical features of patients with IBLC-B and IBLC-T then were compared. Furthermore, their survival was also compared with that of a series composed of 47 patients with phenotypically

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confirmed diffuse large B-cell lymphoma, including immunoblastic lymphoma (B-NHL), but excluding those with clear-cell morphologic characteristics.[14] Survival curves were calculated with the use of the life-table method, and the curves were compared using the log-rank test.

Histopathology

Morphologic evaluation was performed in a blinded manner without information regarding the phenotypic or molecular genetic features of the cases. The evaluation focused on the characteristic histopathologic features of PTCL and included the following cytologic characteristics: monomorphism or polymorphism of the neoplastic cells; pattern of infiltration; background cells and structures such as eosinophils, plasma cells, postcapillary venules and compartmentalization of the neoplastic cells by reticulin fibers. Polymorphism was defined as a feature composed of a spectrum of transformed cells.[6,8] The mitotic rate was determined by counting the number of mitotic cells in 10 high-power fields (HPFs). Cases with a mean value of five or more mitotic cells per HPF (0.16 mm^2) were considered to have frequent mitoses. The amounts of necrosis and fibrosis also were evaluated.

Phenotypic Analysis

The phenotypes of the lymphomas were determined on snap-frozen tissue in 27 cases and on paraffin-embedded tissue in 7 cases in which frozen tissue was not available. Immunohistochemical studies were performed using the avidin–biotin–peroxidase complex (ABC) method.[15] The primary antibodies used and their sources are listed in Table 1. Biotinylated secondary antibodies and ABC were obtained from Vector Laboratories (Burlingame, CA), and antibody localization was demonstrated by incubation of the sections with 3,3'-diaminobenzidine–H_2O_2 solution. The sections then were counterstained with methyl green or hematoxylin and examined with a light microscope. In cases in which only paraffin-section immunohistochemical studies could be performed, a B-cell phenotype was defined as positive membrane staining of the neoplastic cells for L26 antigen along with negative staining for CD45RO (UCHL-1) antigen, with the reverse being true for cases with a T-cell phenotype.[16,17]

Molecular Genetic Analysis

High molecular weight DNA was prepared from frozen tissue of three cases of IBLC-B and five cases of IBLC-T. Three 10-μg aliquots of DNA from each case were digested

TABLE 1. PHENOTYPIC AND GENOTYPIC FEATURES OF CLEAR-CELL IMMUNOBLASTIC LYMPHOMA

Features	Sources of Antibodies	IBLC-B (n = 16)	IBLC-T (n = 18)
Phenotype			
B-cell–related antigens			
Monoclonal surface Ig (κ)	DK	7/12*	0/15
Monoclonal surface Ig (γ)	DK	2/12	0/15
Surface IgM	DK	7/12	0/6
Surface IgG	DK	1/12	0/6
Surface IgM + IgD + IgG	DK	1/12	0/6
Surface IgM + IgG + IgA	DK	1/12	0/6
Cytoplasmic Ig	DK	0/14	ND
CD19 (Leu-12)	BD	6/7	0/12
CD20 (B1)	CI	8/11	0/8
CD21 (B2)	CI	0/5	0/4
CD22 (Leu-14)	BD	8/9	0/11
CD10 (CALLA)	BD	2/9	0/6
L26	DK	4/4	0/3
T-cell–related antigens			
CD5 (Leu-1)	BD	0/10	8/13
CD2 (Leu-5)	BD	0/9	11/14
CD3 (Leu-4)	BD	0/10	10/14
CD7 (Leu-9)	BD	0/3	2/7
CD4 (Leu-3a)	BD	0/12	10/15
CD8 (Leu-2a)	BD	0/12	3/15
CD1 (Leu-6)	BD	0/2	0/8
CD45RO (UCHL-1)	DK	0/4	3/3
Miscellaneous antigens			
HLA-DR (Ia)	BD	9/9	13/15
CD25 (IL-2R)	BD	3/5	2/4
CD71 (OKT 9)	OS	8/8	10/12
CD30 (Ki-1)	DK	0/4	1/5
Genotype			
Clonal rearrangement of J_H		3/3	0/5
Clonal rearrangement of C_β		0/3	5/5

IBLC-B = clear-cell immunoblastic lymphoma of B-cell type; IBLC-T = clear-cell immunoblastic lymphoma of T-cell type; Ig = immunoglobulin; ND = not done; CALLA = common acute lymphoblastic leukemia-associated antigen; IL-2R = interleukin-2 receptor; J_H = J segment of the immunoglobulin heavy chain gene; C_β = constant region of the β-chain of the T-cell receptor gene; DK = DAKOPATTS (Carpinteria, CA); BD = Becton-Dickinson (Mountain View, CA); CI = Coulter Immunology (Hialeah, FL); OS = Ortho Systems (Raritan, NJ).
* Number of cases with feature/number of cases examined.

with the following restriction enzymes: *Bam*HI, *Eco*RI (Bethesda Research Laboratories, Gaithersburg, MD), and *Hin*dIII (Behringer Mannheim, Indianapolis, IN). DNA fragments then were electrophoresed on 0.8% agarose gel and transferred to nylon filters by Southern blotting. After hybridization to ^{32}P-labeled DNA probes for immunoglobulin (Ig) genes or T-cell receptor genes, filters were exposed to XAR-2® film (Eastman Kodak Company, Rochester, NY), with the use of intensifying screens. DNA probes for the Ig heavy-chain genes (5.4-kb fragment of human genomic DNA-J_H) and the T-cell receptor beta-chain genes (0.4-kb fragment of human complementary DNA-$C_{\beta2}$) were obtained from Oncogene Science (Manhasset, NY). Human placental DNA was used to confirm the germline configuration of DNA fragments. Clonality was determined when nongermline bands were detected in at least two of the three enzyme digests.

RESULTS

Of the 34 cases of IBLC, phenotypic studies revealed that 16 cases were of B-cell type and 18 cases were of T-cell type (Table 1). Of 12 cases of IBLC-B in which frozen tissue was available, 9 cases had monoclonal surface Ig. Heavy chains were positive in ten cases, and seven cases had surface IgM. Heavy chains were not detected in one of nine light chain–positive cases, whereas light chains were not detected in two of ten heavy chain–positive cases. Cytoplasmic Ig was not detected in any of the 14 cases examined. The neoplastic cells in most of the cases studied by frozen-section immunohistochemistry also were positive for one or more B-cell–related antigens, such as CD19, CD20, or CD22, whereas they were negative for all T-cell–related antigens. In the four cases in which only paraffin-embedded tissue was available, the neoplastic cells were positive for L26 antigen and negative for CD45RO antigen. Scattered small lymphoid cells with T-cell–related antigens were admixed with the proliferating large cells in most of the cases of IBLC-B. Frozen-section immunohistochemical studies performed on 15 cases of IBLC-T showed ten cases to be of a helper–inducer phenotype and three cases to be of a suppressor–cytotoxic phenotype. HLA-DR and CD71 were expressed on most of both IBLC-B and IBLC-T cases.

Molecular genetic analysis was performed in eight cases of IBLC. In three cases, including one surface Ig–negative case, clonal rearrangements of the Ig heavy chain genes were demonstrated, whereas five cases showed clonal rearrangements of the T-cell receptor beta-chain genes. There was no discrepancy between the phenotypic and genotypic findings in any of these eight cases.

The cytologic features of the neoplastic clear cells in IBLC-B were similar to those in IBLC-T, although the cells were less likely to be polymorphic in IBLC-B (Figs. 1 and 2; Table 2). The cells had medium-sized to large, centrally located nuclei that were round or slightly oval in shape in most cases in both groups. Multilobated nuclei were seen in one case of IBLC-B. Nucleolar features, such as size, number per nucleus, and location, varied considerably, even in the monomorphic cases. A higher mitotic rate, interfollicular proliferation, infiltration by eosinophils, and an increase in postcapillary venules with endothelial hyperplasia were seen more often in IBLC-T. Necrosis and fibrosis were more extensive in IBLC-B (Table 2).

The clinical features of the 34 patients are summarized in Table 3. Follow-up information was available for 31 patients. Twenty-eight were treated with various clinical protocols, whereas the remaining 3 did not receive radiation therapy or chemotherapy. Most of the patients with IBLC-B were female, whereas most of those with IBLC-T were male. The mean and median ages at the time of diagnosis for the patients with IBLC-T were lower than for those with IBLC-B, and patients with IBLC-T had more advanced disease at the time of diagnosis than those with IBLC-B. The sites of involvement in both cell types were predominantly nodal, and only 1 of 16 patients with IBLC-B and 1 of 18 patients with IBLC-T had mediastinal masses. The median and predicted three-year actuarial survival rates were slightly worse for patients with IBLC-B than for those with IBLC-T. However, there were no statistically significant differences in the actuarial survival rates of patients with IBLC-B, IBLC-T, or diffuse large B-NHL other than IBLC-B (Fig. 3).

DISCUSSION

Among 34 cases of IBLC, the neoplastic cells were confirmed to be of B-cell type in 16 cases by phenotypic and, in some cases, molecular genetic analyses. This finding was rather surprising because clear-cell morphologic characteristics generally have been considered to be a characteristic feature of PTCL.[5-13] The phenotypic features of IBLC-B (CD19 positive, CD20 positive, CD22 positive, HLA-DR positive, CD10 negative, and CD21 negative) suggest that the neoplastic cells may have originated from extrafollicular B cells, which is in accordance with the concept of immunoblastic lymphoma.[8] Based on the pattern of surface Ig expression, 7 of 12 cases of IBLC-B were characterized as having immature phenotypes because of the expression of only surface IgM, whereas 3 cases had a more mature phenotype with the expression of surface IgG with or without other isotypes.[18] It is interesting that plasmacytoid B-immunoblastic lymphoma also may have either an immature or a mature phenotype.[18] The absence of cytoplasmic Ig in the cases of IBLC-B suggests that the neoplastic cells were at an earlier stage of maturation than typical B-immunoblasts.

The pattern of expression of several activation-associated antigens[19] in IBLC-B also may be important in explaining the histogenesis of clear cells. In the process of B-cell activation by an antigen or mitogen, sequential alterations of the membrane structure characterized by the expression of CD25, an increase in the density of HLA-DR, and the acquisition of CD71 are observed before DNA synthesis,[19-21] whereas the expression of CD30 does not occur until late in the cell cycle.[22] In IBLC-B, CD25 was present in three of five cases and CD71 was present in all eight cases examined, but none of four cases expressed CD30 (Table 1). This pattern of activation-associated antigens on the neoplastic cells of IBLC-B suggests that the cells are in the relatively early phase of the cell cycle. Although CD25 and CD71 are expressed in many high-grade lymphomas and are not specific for any one

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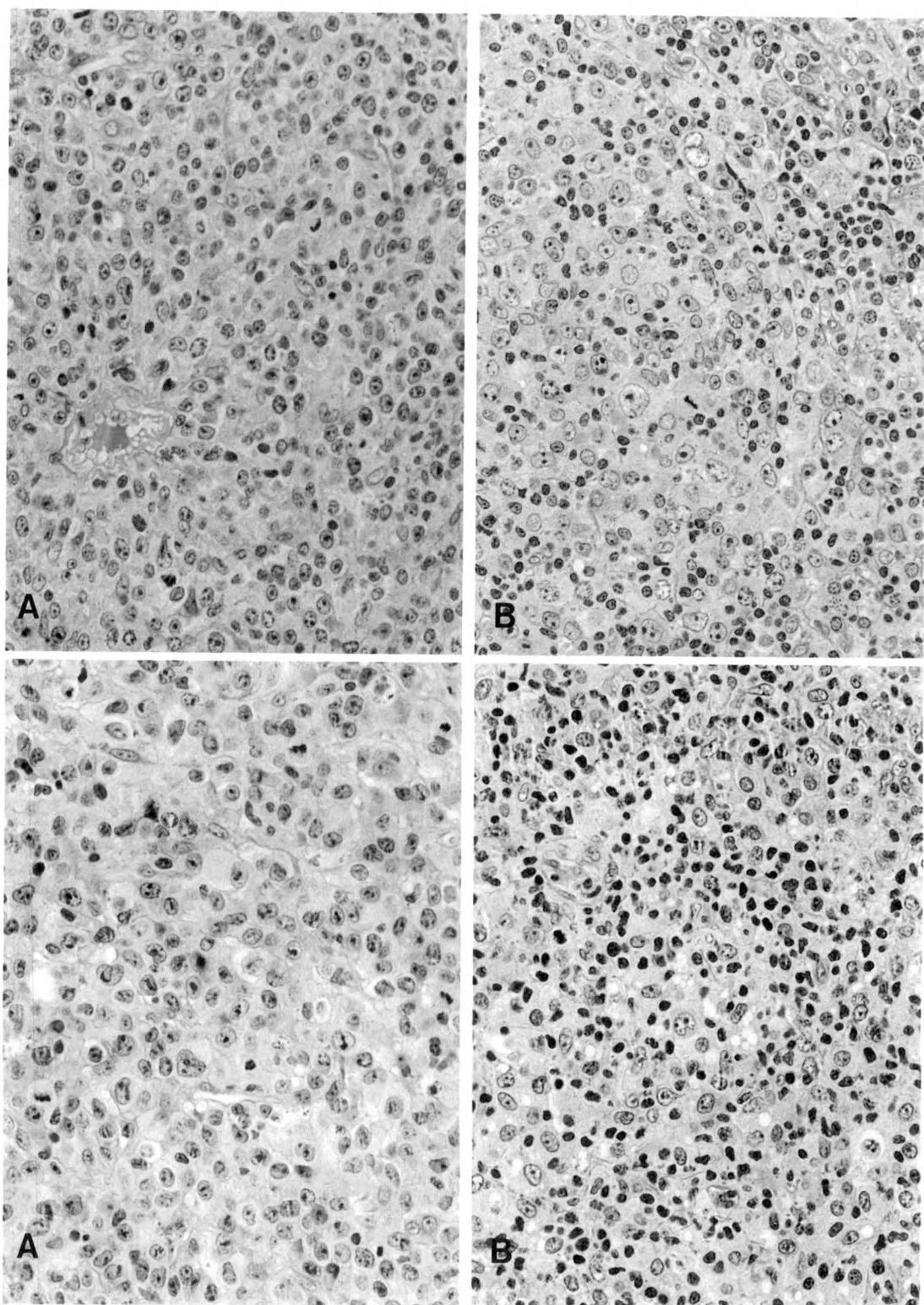
FIG. 1 (*upper*). Histologic features of two cases of IBLC-B. *A.* Fairly uniform population of neoplastic cells with round to slightly oval nuclei, centrally placed nucleoli, and abundant clear cytoplasm. This lymphoma was classified as monomorphic. Hematoxylin and eosin (×720). *B.* In contrast to Figure 1*A*, the proliferating cells in this case show prominent nuclear variability and the lymphoma was classified as polymorphic. Hematoxylin and eosin (×720).

FIG. 2 (*lower*). Histologic features of two cases of IBLC-T. Similar to Figure 1, both monomorphic (*A*) and polymorphic (*B*) lymphomas consisting of cells with abundant clear cytoplasm are shown. Hematoxylin and eosin (×720).

TABLE 2. HISTOPATHOLOGIC FEATURES OF CLEAR-CELL
IMMUNOBLASTIC LYMPHOMA

Features	IBLC-B (n = 16)	IBLC-T (n = 18)
Morphologic characteristics of the neoplastic cells		
Polymorphic	8 (50)*	14 (78)
Frequent mitoses†	7 (44)	12 (67)
Pattern of proliferation		
Interfollicular	0 (0)	7 (39)
Background cells/structures		
Presence of eosinophils	2 (13)	10 (56)
Increase in mature plasma cells‡	9 (56)	12 (67)
Increase in postcapillary venules	3 (19)	12 (67)
Compartmentalization by reticulin	12 (75)	17 (94)
Extensive fibrosis	9 (56)	3 (17)
Extensive necrosis	6 (38)	2 (11)

IBLC-B = clear-cell immunoblastic lymphoma of B-cell type; IBLC-T = clear-cell immunoblastic lymphoma of T-cell type.
* Number of cases with feature; percentage in parentheses.
† Number of cases with five or more mitotic cells per high-power field.
‡ Monoclonal plasma cells were not found.

FIG. 3. Predicted actuarial survivals of patients with clear-cell immunoblastic lymphoma of B-cell type (IBLC-B) and T-cell type (IBLC-T), and other diffuse large B-cell lymphoma, including immunoblastic lymphoma (B-NHL), but excluding those with clear-cell morphologic characteristics.

type of lymphoma, the pattern of the expression of surface and cytoplasmic Ig and activation-associated antigens in our cases supports the view of Schneider and associates,[8] who have described areas composed of clear cells in otherwise typical B-immunoblastic lymphoma and observed that clear cells may be the predominant cell type in some cases. These authors[8] suggested that clear cells in B-immunoblastic lymphoma are less mature plasmacytoid cells that are in the S-phase of the cell cycle. The mechanism of formation of clear cells in PTCL is unknown.[9] However, it should be remembered that activation-associated antigens similarily expressed in our cases of IBLC-T, as well as cases of PTCL reported by others.[10,23] HLA-DR, which is considered to be an activation-associated antigen on T cells, also was positive in most IBLC-T cases. These find-

TABLE 3. CLINICAL FEATURES OF CLEAR-CELL
IMMUNOBLASTIC LYMPHOMA

Features	IBLC-B (n = 16)	IBLC-T (n = 18)
Age (years)		
Mean/median	62/62.5	46/43
Range	21–83	7–91
Male/female	6/10	14/4
Nodal/extranodal	13/3	16/2
Stage*		
I + II	8	5
III + IV	7	11
Survival*		
Median (months)	16	24
Actuarial 3-year (%)	20	38

IBLC-B = clear-cell immunoblastic lymphoma of B-cell type; IBLC-T = clear-cell immunoblastic lymphoma of T-cell type.
* Information on one IBLC-B and two IBLC-T cases was not available.

ings suggest that clear cells of both B- and T-cell types may be generated by a common mechanism.

Histologically, there were no apparent differences in the morphologic characteristics of the clear cells in IBLC-B and IBLC-T, although polymorphic composition of the neoplastic cells, interfollicular proliferation, a higher mitotic rate, infiltration by eosinophils, and increased postcapillary venules appear to be indicators of IBLC-T, as has been suggested previously.[4–9,11–13] PTCL can be classified into the following major histologic types: atypical small lymphocytic type, mixed-cell type, and large cell type.[5,12,13] Although additional terms such as lymphoepithelioid cell type[7] and immunoblastic type[11] are used occasionally, they also can be included in one of the three major types.[13] Another classification for PTCL has been proposed recently[9] and integrated into the updated Kiel classification for NHL.[24] However, clear-cell morphology is not a specific feature of the large cell or immunoblastic types and may be seen in the atypical small lymphocytic and mixed-cell types as well.[5,7,9,11–13] The nonspecificity of clear-cell morphologic characteristics is also true for the phenotypes of PTCL, in which the feature was seen in both helper–inducer and suppressor–cytotoxic phenotypes in our cases of IBLC-T, as well as those of PTCL reported by others.[9,23] We conclude that clear-cell morphologic characteristics alone cannot be used to differentiate any one of the histologic types or phenotypes of PTCL from the others, nor is it specific for PTCL of the large cell type.

Several differences in the clinical features of patients with IBLC-B and those with IBLC-T were found in the

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current series. The median age of the patients with IBLC-B (62.5 years) was similar to that of patients with diffuse large B-NHL.[25] However, patients with IBLC-T were younger at the time of diagnosis (median age, 43 years). In general, PTCL occurs in the sixth or seventh decade of life[7,11,26,27]; this is also true for patients with PTCL of the large-cell type.[7,25,26] Men were affected more often by IBLC-T, as has been described in most series of PTCL.[7,11,25,26,28] In contrast, the female predominance among patients with IBLC-B appears to be unusual, and this finding is further discussed below. Patients with PTCL[7,11,26-29] and those with diffuse large B-NHL[14,25] often have advanced disease (stage III or IV) at the time of diagnosis. In our series, 11 of 16 patients with IBLC-T had advanced disease, whereas more than half of the patients with IBLC-B had localized disease (stage I or II). However, the median survival lengths and actuarial three-year survival rates were not significantly different among the patients with IBLC-B (16 months and 20%, respectively), those with IBLC-T (24 months and 38%, respectively), and those with diffuse large B-NHL other than IBLC (17 months and 30%, respectively; Fig. 3). The younger age of our patients with IBLC-T may explain the slightly better survival rates associated with IBLC-T *versus* IBLC-B. In the literature, the median survival and actuarial three-year survival for patients with PTCL range from 11 months and 20% to 43 months and 57%, respectively,[26-29] and for those with diffuse large B- or non-T-NHL, they ranged from 17 months and 30% to 48 months and 52%, respectively.[14,25,27] Thus, clear-cell morphologic characteristics do not appear to be a prognostic indicator with regard to survival among patients with diffuse large cell lymphoma.

Although NHL is generally more common in men, 10 of 16 patients with IBLC-B reported herein were women. A female predominance also has been reported for mediastinal large B-cell lymphoma.[30-32] Furthermore, this latter lymphoma has been characterized by several clinicopathologic features that also are seen in IBLC-B, including diffuse proliferation of large lymphoid cells with abundant clear cytoplasm, extensive fibrosis, a phenotype consistent with extrafollicular B cells (CD19 positive, CD20 positive, HLA-DR positive, CD10 negative, and CD21 negative), and a relatively unfavorable prognosis.[30-32] However, primary mediastinal involvement, a much younger age of onset (median patient age, 25-32 years), and the frequent lack of surface Ig on the neoplastic cells appear to be rather distinctive features of mediastinal large B-cell lymphoma.[30-32] Mediastinal involvement was seen in only one of our cases of IBLC-B, and surface Ig was detected in 10 of 12 cases in our series.

In conclusion, the current study suggests that IBLC-B may be more common than previously believed. Our study also indicates that clear-cell morphologic characteristics are not a reliable predictor of a T-cell type, nor are they specific for one type of NHL. We believe that this latter finding is important in regard to the histopathologic classification of NHL. Unlike the International Working Formulation,[3] recently proposed classifications for PTCL do not include a separate category or subcategory for cases with clear-cell morphologic characteristics[7,9,13] because clinical features correlating with the presence of clear cells have not been identified.[9] Our current data also support this view.

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Original Article

Non-Hodgkin's Lymphomas of Nasal Cavity and Paranasal Sinuses

An Immunohistochemical Study

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The authors studied the immunophenotype of nine sinonasal lymphomas using a panel of monoclonal antibodies that react with fixed, paraffin-embedded material (EMA, CAM 5.2, CD45, CD37 [MB-1], MB-2, L-26, CDw75 [LN-1], CD45RA [4 KB-5], CD43 [MT-1], and CD45RO [UCHL-1]). There were seven men and two women, with a mean age of 64 years (range, 9–89 years) and median age of 56 years. Three tumors were limited to the nasal cavity, and the other six had multiple sites of involvement, including the nasal cavity (five), antrum (six), ethmoid (two), orbit (two), and hard palate (one). Histologically, one was a lymphoblastic lymphoma (LBL), one was small cleaved-cell lymphoma (SCCL), three were mixed-cell lymphomas (MCLs), and four were large cell lymphomas (LCLs). Four cases were T-cell lymphomas (one SCCL, three MCLs), four were B-cell neoplasms (four LCLs), and one was of uncertain lineage (LBL).

Angioinvasion, coagulative necrosis, and epitheliotropism were seen in the T-cell lymphomas. Extranasal dissemination was seen in four cases: one LBL that involved the lymph nodes, skin, and testes 15 months after diagnosis; one B-LCL that involved the skin 9 months after diagnosis; and one B-LCL and one T-MCL that involved the gastric mucosa and lung simultaneously with nasal presentation. This study shows a higher predominance of B-cell lymphomas in the sinonasal region than previously reported in Oriental populations. However, the T:B ratio of these lymphomas is still greater than that observed for primary lymph node–based neoplasms. (Key words: Non-Hodgkin's lymphomas; Sinonasal lymphomas; Extranodal lymphomas; Immunophenotype; Immunohistochemistry) Am J Clin Pathol 1991; 96:184–190

Non-Hodgkin's lymphomas of the nasal cavity and paranasal sinuses occur infrequently. They constitute 0.44–2.2% of all extranodal lymphomas and 6.4–13% of extranodal lymphomas of the head and neck.[1-5] Several studies have defined the clinicopathologic characteristics and biologic behavior of these lymphomas.[6-11] Immunologic studies of lymph nodal and extranodal lymphomas now permit classification of these neoplasms according to their B- or T-cell lineages. Although some morphologic studies of nasal and paranasal sinus lymphomas suggest that these neoplasms are of B-cell lineage,[8-10] most

of the immunohistochemical reports have demonstrated a predominance of the peripheral T-cell phenotype.[12-17] These studies were performed predominantly in populations from the Far East, however, where T-cell lymphomas are more common than in western populations. Immunophenotypic studies of sinonasal lymphomas in western populations—although few in number—have demonstrated a predominance of B-cell[5,18] or T-cell[19,20] lymphomas. It is unclear whether the T-cell predominance in nasal and paranasal lymphomas is a geographic difference or a specific characteristic of the neoplasms of this anatomic region.

Recently, monoclonal antibodies that recognize lymphoid antigens in fixed and paraffin-embedded material have been described. These reagents provide a reliable method of phenotyping most lymphomas[21-28] and are useful for the study of retrospective material when no fresh material is available. In general, they allow a good correlation between phenotype and cell morphologic characteristics.

In this study, we analyzed the immunophenotypes of the nasal and paranasal lymphomas observed in our de-

partment and correlated the phenotypes of the lymphomas with their clinicopathologic characteristics.

MATERIALS AND METHODS

Ninety-eight malignant tumors of the nasal cavity and paranasal sinuses diagnosed at our hospital from 1974 to 1988 were reviewed. Among them, ten malignant lymphomas with initial presentation in this area were identified. One of these lymphomas was excluded because no appropriate paraffin blocks were available for immunohistochemical study. Primary nasopharyngeal tumors were not included in this study.

The clinical records of each patient were reviewed to determine the clinical symptoms and the duration, location, and stage of tumors; modes of therapy; and follow-up. Histologic sections of all primary tumors were reviewed. The lymphomas were classified according to the Working Formulation[29] and the updated Kiel classification.[30,31] In three of four patients with extranasal dissemination, biopsy specimens from skin, testes, lymph node, and stomach also were examined.

Specimens were fixed in 10% buffered formalin and, in some cases, in B5, and embedded in paraffin. Sections were stained with hematoxylin and eosin, Giemsa, periodic-acid Schiff (PAS), and orcein.

Serial sections were cut, deparaffinized in xylene, hydrated, and immunostained with the avidin–biotin–peroxidase complex (ABC) technique,[32] using the monoclonal antibodies that are listed in Table 1. The sections were incubated with the primary antibody for one hour at room temperature, washed in phosphate-buffered saline (PBS), and incubated with biotinylated horse antimouse immunoglobulins (DAKO, Copenhagen, Denmark) for 30 minutes, followed by ABC (DAKO) for 40 minutes. The peroxidase reaction was developed with a solution of 3,3'-diaminobenzidine and H_2O_2. Finally, the sections were counterstained with hematoxylin. In two cases, fresh-frozen tumor tissue was also available for immunohistochemical study. In these cases, cryostat sections were cut, dried, and fixed in cold acetone for 10 minutes. Immunostaining was performed with the same ABC technique and with the use of the following monoclonal antibodies: CD19 (Leu-12; Becton-Dickinson [BD], Mountain View, CA), CD22 (To 15; DAKO), CD2 (Leu-5; BD), CD3 (Leu-4; BD), CD4 (Leu-3a; BD), CD5 (Leu-1; BD), CD7 (Leu-9; BD), CD8 (Leu-2; BD), CD25 (IL-2 receptor; BD), CD35 (To 5; DAKO), CD57 (Leu-7; BD), and HLA-DR (BD).

RESULTS

The clinicopathologic characteristics of the patients are summarized in Table 2. In this series, there were seven men and two women. Ages ranged from 9 to 89 years, with a mean of 64 years and a median of 56 years.

Six patients presented with nasal obstruction, two with rhinorrhea, and two with epistaxis. Five patients had facial pain and headache. Unilateral proptosis and paresthesia of the cheek were reported in two and one patients, respectively. Only one patient (patient 1) had fever and weight loss. The duration of symptoms before diagnosis ranged from 4 to 40 weeks (mean, 4.3 months; median, 2 months).

When it was diagnosed, lymphoma was limited to the nasal cavity in three patients. The other six patients had multiple sites of involvement, including the nasal cavity in five patients, the antrum in six, the ethmoid in two, the orbit in two, and the hard palate in one.

Extrasinonasal involvement was evident in four patients (44%). In two patients, distant involvement of the lung and stomach was detected during staging maneuvers; in one patient (patient 6), cutaneous nodules developed on the lower extremities 9 months after diagnosis, and, in the other patient (patient 1), involvement of the cervical lymph node, testes, and trunk skin developed 15 months

TABLE 1. MONOCLONAL ANTIBODIES REACTIVE ON PARAFFIN-EMBEDDED MATERIAL USED IN THIS STUDY

Antibody (cluster of differentiation)	*Immunoreactivity*	*Source*
EMA	Epithelial cells and some lymphoid cells	DAKOPATTS (Copenhagen, Denmark)
CAM 5.2	Low molecular weight keratins, epithelia	Becton-Dickinson (Mountain View, CA)
PD 7/26 + 2B11 (CD45)	Leukocyte common antigen	DAKOPATTS
L-26	B-lymphocytes	DAKOPATTS
MB-1 (CD37)	B-lymphocytes, some mature T-lymphocytes, macrophages and monocytes	BioGenex (San Ramon, CA)
MB-2	B-lymphocytes	BioGenex
LN-1 (CDw75)	B-lymphocytes, particularly follicular center cells	BioGenex
4 KB-5 (CD45RA)	B-lymphocytes and some T-Cell subsets	DAKOPATTS
MT-1 (CD43)	T-lymphocytes, histiocytes, myeloid cells, erythroid precursors, megakaryocytes	BioGenex
UCHL-1 (CD45RO)	T-lymphocytes, macrophages, myeloid cells	DAKOPATTS

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TABLE 2. CLINICAL FINDINGS, DIAGNOSES, AND FOLLOW-UP OF NINE PATIENTS WITH SINONASAL LYMPHOMAS

Case	Age/Sex	Initial Site of Disease	Involvement of Distant Sites/Time (months)	Diagnoses (WF)	Therapy	Status Time
1	9/M	Right nasal cavity	Cervical lymph node Skin, testes (15 M)	LBL-U	Radiotherapy Chemotherapy	DOD/24 M
2	50/M	Nasal cavity Right antrum Right ethmoid	No	SCCL-T	Radiotherapy CHOP	ANED/34 M
3	58/M	Left nasal cavity	No	MCL-T	Radiotherapy	ANED/80 M
4	41/M	Left nasal cavity Left nasal antrum Left ethmoid	Lung (synchronic)	MCL-T	CHOP	DOD/3 M
5	89/M	Right nasal cavity Right antrum	No	MCL-T	CHOP	AWD/2 M
6	82/M	Left nasal cavity	Skin (9 M)	LCL-B	Radiotherapy	DOD/24 M
7	56/M	Left nasal cavity Left antrum	No	LCL-B	Radiotherapy CHOP	ANED/23 M
8	51/F	Right antrum Right orbit Hard palate	Stomach (synchronic)	LCL-B	Radiotherapy Promace-Cytabom	ANED/13 M
9	73/F	Left nasal cavity Left antrum Left orbit	No	LCL-B	CHOP	ANED/6 M

M = male; F = female; WF = Working Formulation; CHOP = cyclophosphamide, adriamycin®, vincristine, prednisone; Promace-Cytabom = cyclosphosphamide, adriamycin, vincristine, meth-otrexate, bleomycin, VP-16, cytosine-arabinoside, prednisone; DOD = dead of disease; ANED = alive, no evidence of disease; AWD = alive with disease; U = uncertain lineage.

after diagnosis. The extranasal disease was documented histologically in skin (two cases), lymph node, testes, and stomach.

Treatment regimens varied (Table 2). Most of the patients had received local radiation therapy and/or chemotherapy. Eight patients had a good response; in seven cases the disease disappeared, whereas in one patient the tumoral mass was reduced after two months of chemotherapy (patient 5). Two patients died with generalized disease (patients 1 and 6). In case 8, a second biopsy from the right antrum and stomach disclosed no evidence of disease after treatment. No response to treatment was observed in one patient (patient 4), who died three months after diagnosis.

Histopathologic and Immunohistochemical Findings

All of the lymphomas showed a diffuse growth pattern. Four cases had a predominance of large cells with large, round, or oval nuclei and several nucleoli located at the nuclear membrane (Fig. 1). All of these cases showed a variable presence of immunoblasts and large and small cells with cleaved nuclei. These cases were classified as large cell lymphomas (LCLs) or centroblastic lymphomas of polymorphous type. Three cases contained a diffuse infiltrate composed of medium-sized cells with irregular nuclei. Small and large cells with angulated and irregular nuclei also were present in variable numbers. These cases could be classified as diffuse mixed-cell lymphoma (MCL) or mixed size pleomorphic lymphoma (Fig. 2). In one case, the predominant cells were small lymphocytes with irregular nuclei, condensed chromatin, and inconspicuous nucleoli. Cytoplasm was very scanty, and there were very few large cells with a blastic appearance. This case was classified as diffuse small cleaved cell lymphoma (SCCL) or small cell pleomorphic lymphoma. Finally, one case (case 1) consisted of sheets of medium-sized cells with round nuclei and was diagnosed as lymphoblastic lymphoma (LBL).

A variable degree of coagulative necrosis was observed in three cases (two MCLs and one SCCL). In each case, tumoral invasion of vascular structures—with complete occlusion of some vessels—was a prominent feature. Neurotropic infiltration by tumor cells also was seen in one of these cases (case 4) (Fig. 3). Neither angioinvasion nor coagulative necrosis was detected in the four LCLs, one MCL, or one LBL. Invasion of epithelial structures, overlying mucosal epithelium, mucosal glands, and skin was observed in two MCLs (cases 3 and 5). However, invasion of the mucosa was difficult to evaluate because of the extensive ulceration and superficial necrosis in most cases and because of the small size of some biopsy specimens. In three LCLs in which the nasal mucosa was preserved, the epithelial layer was not invaded by tumor cells. A reactive inflammatory infiltrate of small, normal-appearing lymphocytes, plasma cells, granulocytes, and histiocytes was seen predominantly in MCL and SCCL.

The histologic appearance of lymphomas in extrasinonasal sites was similar to that observed in the sinonasal tumors. The gastric biopsy specimen from one case showed a partial substitution of gastric mucosa by sheets
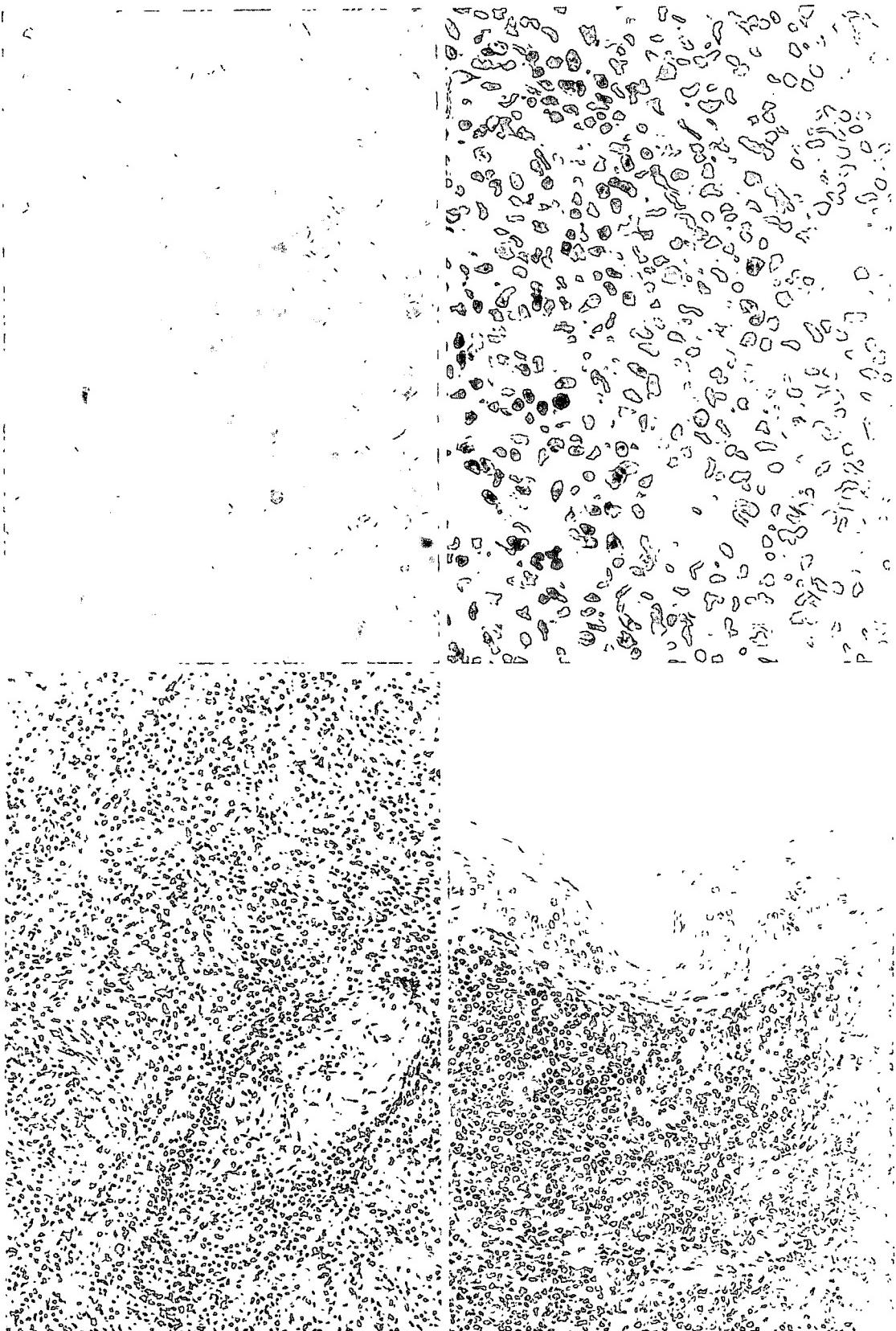
FIG. 1 (upper, left). Diffuse large cell lymphoma involving left nasal cavity, case 6. Hematoxylin and eosin (×400).

FIG. 2 (upper, right). Diffuse infiltrate composed of small and large cells with irregular nuclei, case 4. Hematoxylin and eosin (×250).

FIG. 3 (lower, left). Vascular and perineural infiltration by atypical T lymphocytes, case 4. Hematoxylin and eosin (×100).

FIG. 4 (lower, right). Skin involvement without epidermotropism in a B-LCL, case 6. Hematoxylin and eosin (×100)

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TABLE 3. IMMUNOHISTOCHEMICAL FINDINGS OF NINE SINONASAL LYMPHOMAS

Case	Histologic	CD45	CD45RA	MB-1	MB-2	L-26	LN-1	CD45RO	MT-1	Conclusion
1	LBL	+	−	−	+	−	−	±	+	U
2	SCCL	+	−	−	−	−	−	++	±	T
3	MCL	+	−	−	ND	−	−	+++	ND	T
4	MCL	+	−	−	−	−	−	+++	+	T*
5	MCL	+	+	ND	ND	−	ND	−	ND	T†
6	LCL	+	ND	++	ND	+	ND	−	−	B
7	LCL	+	++	+++	+++	+++	+++	−	−	B
8	LCL	+	++	+++	++	++	+++	−	−	B
9	LCL	+	++	ND	ND	++	ND	−	−	B

ND = not done; U = uncertain lineage.
* On frozen-section study, case 4 was CD2, CD3, and CD4 positive and CD7, CD5, and CD8 negative.

† On frozen-section study, case 5 was CD2, CD3, CD7, CD8, and HLA-DR positive and CD5 and CD4 negative.

of large and atypical lymphoid cells without evidence of lymphoepithelial lesions. Skin involvement in cases 1 and 6 showed a dense infiltrate in the dermis without epidermal invasion (Fig. 4).

The immunopathologic findings are summarized in Table 3. No reactivity for CAM 5.2 and EMA was observed in any case, and all of the cases were positive for leukocyte common antigen (CD45). Four cases had a B-cell phenotype (Fig. 5), whereas four were interpreted as T-cell neoplasms (Fig. 6). The LBL was of uncertain lin-

eage. The four B-cell tumors were LCLs with polymorphic features, whereas the T-cell lymphomas were SCCL (one case), or MCL (three cases). The immunohistochemical pattern of extrasinonasal lesions was identical to that of the primary tumors.

The immunohistochemical study performed on fresh-frozen material in cases 4 and 5 disclosed an anomalous phenotype in both cases: CD3 and CD2 antigens were expressed, CD7 was lost in case 4, and CD5 was absent in both cases. One case expressed CD4, whereas the other
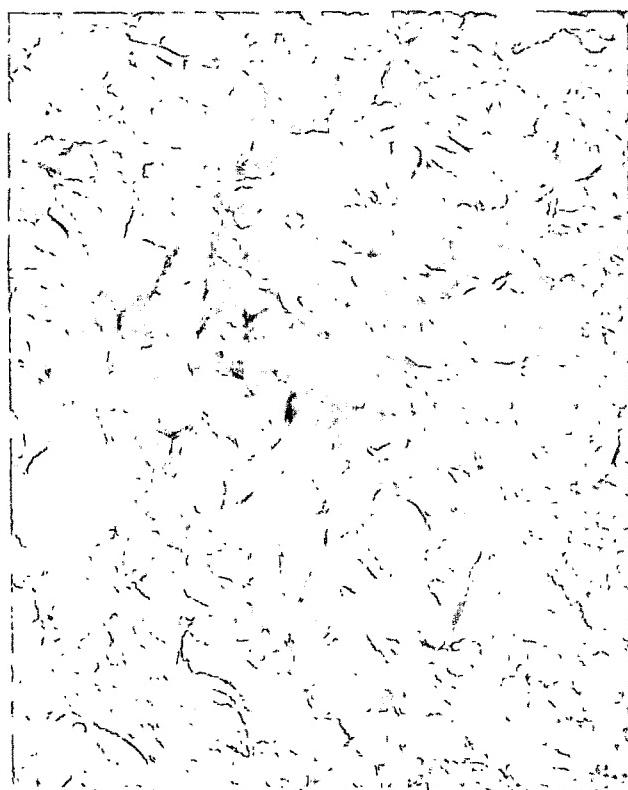
FIG. 5 (*left*). L-26 immunostaining of a large cell lymphoma, showing a membranous pattern, case 6. Immunoperoxidase-hematoxylin (×400).
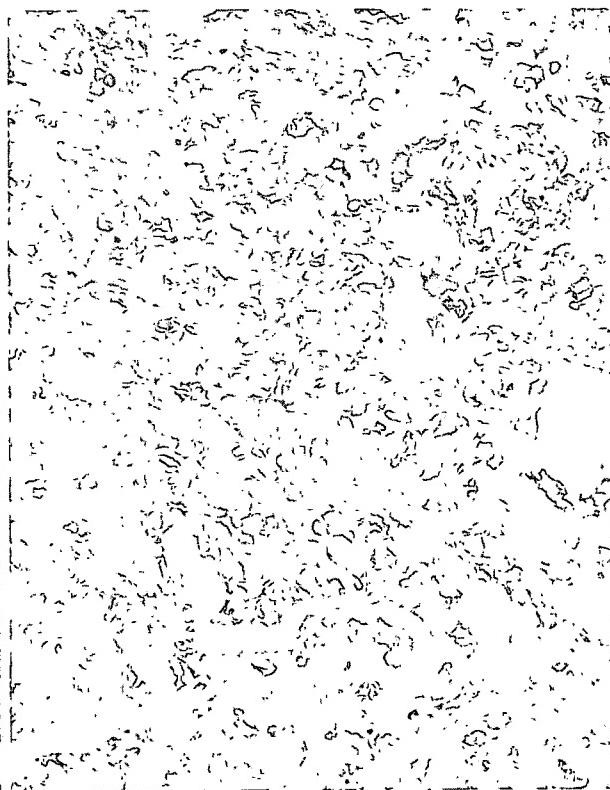
FIG. 6 (*right*). UCHL-1 immunostaining of a mixed-cell lymphoma, case 4. Immunoperoxidase-hematoxylin (×250).

case had an activated (HLA-DR positive) CD8 phenotype. This case was CD45RA positive and CD45RO negative on paraffin sections.

DISCUSSION

Lymphomas of the sinonasal region are not a "single disease." In a retrospective study of 98 malignant tumors of the nasal cavity and paranasal sinuses, we found ten lymphomas. This incidence of 10% is similar to the figures of 5.8–8% reported previously.[4,6,9] The clinical presentation and location of lymphomas investigated in the current study are similar to those observed in other large series.[6–10] Most of the studies of sinonasal lymphomas have analyzed the clinicopathologic characteristics of these neoplasms. Their immunophenotypes have been assessed less often. In contrast to non-Hodgkin's lymphomas of Waldeyer's ring, which express a B-cell phenotype,[14,33] most of the previously characterized nasal lymphomas have been found to be peripheral T-cell neoplasms.[12–17] These studies, however, were performed mainly on Oriental populations, in which T-cell lymphomas predominate over B-cell types. The presence of four B-cell neoplasms (44%) in our series and the predominance of B-cell types in other western studies[5,18] suggest that the phenotype of sinonasal lymphomas is related in part to geographic differences, with a higher ratio of B-cell lineage in western populations than in Oriental populations; however, we also found a high proportion of T-cell lymphomas (five of nine). Other European studies have noted that almost all lymphomas presenting as lethal midline granuloma were peripheral-T-cell lymphomas.[19,20] These findings suggest that, even in western populations, the T:B ratio of sinonasal lymphomas is higher than that observed for lymph node neoplasms.

The immunologic characterization of sinonasal lymphomas shows a good correlation between the immunophenotype and histologic characteristics. In our study, angioinvasion, coagulative necrosis, epitheliotropism, and inflammatory infiltrate only were associated with a T-cell phenotype. Three neoplasms with these characteristics were reactive for the pan-T markers UCHL-1 and MT-1 and negative for all B antigens. Case 5 was CD45RA positive in fixed-paraffin material and had negative results with other pan-B and pan-T-cell markers. This case had a T-cell–activated CD8-positive suppressor/cytotoxic phenotype on frozen-section analysis. Previous studies have demonstrated that CD45RA antibodies react with some normal and malignant T cells, mainly of CD8 subset.[34,35] The LBL in our series had positive results with MT-1 and MB-2, with weaker staining with UCHL-1. MB-2 has been considered to be a pan-B marker,[22,24,26,28] but it also reacts with some T-LBLs.[27] MT-1 recognizes

T-cell lymphomas as well as some B-cell neoplasms.[24,27,28] Therefore, these results do not allow us to establish clearly the lineage of this lymphoma. LCLs with centroblastic features had a B-cell phenotype. Two cases also were positive with LN-1, an antibody that recognizes an antigen found mainly in large follicular center cells.[21,26] None of the B-cell lymphomas, nor the T-LBL, showed angiotropism, coagulative necrosis, or epitheliotropism.

The T-pleomorphic lymphomas had histologic characteristics of the so-called "polymorphic reticulosis" or "midline malignant reticulosis."[16,36] This entity now is considered to be a malignant lymphoma of T lineage that is related to the angiocentric immunoproliferative lesions.[5,7,16,18,37] Immunophenotypic studies have demonstrated that most of these T-cell proliferations are of T-helper type and that most of them have lost one or more T-cell antigens, as has been observed in nodal peripheral T-cell lymphomas.[38–40] The two cases that we studied on fresh-frozen material also expressed an anomalous T phenotype, with a loss of pan-T antigens (CD5 and CD7). A histiocytic phenotype rarely has been associated with midline lethal granulomas.[20,41]

Previous studies of sinonasal lymphomas have shown a trend for these lymphomas to disseminate outside the nasal and paranasal region, particularly to such extranodal sites as the gastrointestinal tract, skin, lung, brain, bone, and breast.[7,9,11,14,18] Similarly, four of nine patients (44%) in our study presented with extranasal and extranodal disease. Previous studies on T-cell sinonasal lymphomas have demonstrated a tendency toward dissemination, mainly to the lung, skin, and gastrointestinal tract. No information is available regarding the spread of sinonasal B-cell lymphomas. In our study, extranasal involvement by lymphoma was independent of phenotype, because two were of B-cell (stomach and skin) and two were of T-cell type (lung and lymph node; and skin and testes). The morphologic and immunohistochemical features of the extranasal biopsy specimens were similar to those of the primary sinonasal tumors.

Yamanaka and associates[14] have shown that nasal lymphomas were associated with a poorer prognosis than Waldeyer's neoplasms. They suggested that these differences could be related to the phenotype of the tumors because Waldeyer's lymphomas were mainly of B-cell type, whereas nasal neoplasms were T-cell neoplasms. It is not clear, however, whether the phenotype of sinonasal lymphomas can predict their progression. In our series, two of four long-term survivors had T-cell lymphomas.

In summary, Western lymphomas of sinonasal area are more likely to be of B-cell origin than sinonasal lymphomas presenting in Oriental populations. However, the T:B ratio of these lymphomas is still greater than that observed for lymph node neoplasms. Additional studies re-

porting large series from different geographic locations will be necessary if we are to clarify whether or not the relative frequencies of B and T malignant lymphomas are related to geographic variations.

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The Transient Appearance of Small Blastoid Cells in the Marrow after Bone Marrow Transplantation

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Of 14 patients who underwent allogeneic or syngeneic bone marrow transplantation, 6 had a transient appearance of small blastoid cells in the bone marrow after transplantation. Most of these patients (11) had leukemia, although 3 had severe aplastic anemia. The cells were 8–18 μm in diameter and had scant cytoplasm and dense nuclei with smooth, homogeneous chromatin. They often had distinct nuclear clefts. These cells constituted 4.0–21.3% of the total number of bone marrow cells. They were not reactive with peroxidase, alpha-naphtyl butylate esterase, naphthol AS-D chloroacetate esterase, or periodic acid-Schiff stains. Immunocytochemical analysis revealed that the small blastoid cells expressed terminal deoxynucleotidyl transferase, Ia-like, CD19, and CD10 antigens and cytoplasmic mu heavy chains, indicating a precursor B-cell phenotype. CD20 antigen was not expressed on these cells. The data suggest that cytoplasmic mu may be expressed earlier than CD20 antigen in the differentiation of B-cell lineage. The morphologic, cytochemical, and immunophenotypic characteristics did not distinguish these nonneoplastic cells distinctly from leukemic lymphoblastic cells. The increase of small blastoid cells was a transient and self-limited phenomenon, in contrast to that of neoplastic blasts. These cells should be recognized as a common component of the bone marrow of marrow transplant recipients. The significance and role of these cells in immune recovery and hematopoiesis remain uncertain. (Key words: Blastoid cell; Bone marrow transplantation; B-cell; Hematogones) Am J Clin Pathol 1991;96:191–195

The transient appearance of small blastoid cells was recognized in the bone marrow in 6 of 14 patients with leukemia in continued remission and aplastic anemia after allogeneic and syngeneic bone marrow transplantation. These cells had scant cytoplasm and dense nuclei with smooth homogeneous chromatin. They morphologically resembled the lymphoblastic cells observed in patients with L1 leukemia, as categorized by the French–American–British (FAB) classification.[1] These small blastoid cells have been recognized to be a minor component in the bone marrows of normal people.[2] Significant increases in the numbers of these cells have been reported in a variety of disorders, and several other names have been applied to them, according to the conditions of patients or the methods used: hematogones,[2] posttherapeutic lymphoid cells,[3] residual blasts,[4] precursor cells,[5,6] terminal deoxynucleotidyl transferase (TdT)–positive cells,[7,8] common acute lymphoblastic leukemia antigen (CALLA)–positive cells.[9–12] In this report, we present the immunophenotypic features of the small blastoid cells that appeared in the bone marrow of transplant recipients. The significance of these cells also is discussed.

MATERIALS AND METHODS

Patients

Bone marrow transplantation (BMT) was performed in 11 patients with hematologic malignancies in remission or the chronic phase (6 with acute myelogenous leukemia, 4 with acute lymphoblastic leukemia [ALL], and 1 with chronic myelogenous leukemia) and 3 with severe aplastic anemia during the period from June 1985 to August 1988. All marrow donors were HLA genotypically identical sib-

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lings, with two exceptions. One of the latter was a phenotypically identical mother, and the other was an identical twin. A transient appearance of small blastoid cells (more than 4% of the total number of bone marrow cells) was recognized after bone marrow transplantation in 6 of the 14 patients (Table 1). The recipients received 2.5–3.6×10^8 (mean, 3.2×10^8) marrow cells/kg. The clinical assessment and gradation of graft-*versus*-host disease (GVHD) were based on involvement of gut, liver, and skin, as reported by Thomas and associates.[13] All transplant recipients with severe aplastic anemia received a four-day conditioning regimen with daily cyclophosphamide (50 mg/kg) and total lymphoid irradiation (800 cGy). Some transplant recipients with leukemia received a conditioning regimen of cytosine arabinoside (1.4 g/m^2) (four doses on days -6 and -5), cyclophosphamide (60 mg/kg) (two doses on days -4 and -3), and 1,250 cGy fractionated total body irradiation (TBI) from day -2 to day 0 (patients 3–5). Others received a conditioning regimen of busulfan (37 mg/m^2) orally (16 doses on days -9, -8, -7, and -6) in place of TBI (patients 1 and 2). All six patients receiving allogeneic grafts and one patient receiving syngeneic bone marrow transplantation received short term methotrexate (10–15 mg/m^2/day) and continuous infusion of cyclosporine (3 mg/kg/day) as GVHD prophylaxis.

Marrow Examination

When possible, bone marrow aspiration was performed (after informed consent was obtained) every week during the first 35 days after transplantation, two or three times between days 36 and 100 posttransplant, and when needed thereafter. Bone marrow smears were stained with May–Grünwald–Giemsa and various cytochemical staining and immunocytochemical stains. At least 400 small blastoid cells were evaluated in each May-Grünwald-Giemsa–stained bone marrow smear. Bone marrow smears were stained with periodic acid-Schiff (PAS), alpha-naphtyl

butylate esterase, and naphthol AS-D chloroacetate esterase stains.

Immunocytochemical Staining

Immunoalkaline phosphatase methods were used exclusively for immunophenotypic analyses in this study, largely because of the documented superiority of these methods to those using horseradish peroxidase when applied to tissues rich in endogenous peroxidase.[14] One of two different methods was used on each case, depending on the relative efficiency of each commercially available kit in the detection of the antigen of interest: the avidin–biotin alkaline phosphatase complex (ABC) method (VectaStain ABC-AP®, Vector Laboratories, Burlingame, CA)[15,16] or the biotin streptavidin (BSA) alkaline phosphatase complex (StrAviGen AP®, BioGenex Laboratories, Dublin, CA).[17]

Bone marrow smears were air dried and either stained on the day of preparation or stored at -35 °C until staining. Smears were fixed in formol-buffered acetone (200 mg/L Na$_2$HPO$_1$ and 200 mg/L KH$_2$PO$_1$ in 45% acetone and 25% concentrated formalin) for 30 seconds at 4 °C, acetone for 60 seconds at 4 °C, and methanol for 15 minutes at -20 °C. After being washed for 15 minutes in three changes of phosphate-buffered saline (PBS), pH 7.5, a circumscribed area of each smear was overlaid with diluted normal serum from the species from which the secondary antibody was made and placed in a humid chamber for 20 minutes at room temperature. Next, this area was overlaid with monoclonal antibodies diluted in PBS for 30–180 minutes at room temperature or overnight at 4 °C in a humid chamber and washed for 15 minutes in three changes of PBS. Then, the area was overlaid successively with each of the following reagents in a humid chamber for 30–40 minutes at room temperature or at 37 °C: biotinylated horse or goat antimouse IgG antiserum, followed by avidin–alkaline phosphatase complex or streptavidin–alkaline phosphatase complex. Each in-

TABLE 1. CLINICAL CHARACTERISTICS OF PATIENTS

Patient No.	Diagnosis (FAB)	Age/Sex	Acute GVHD	Chronic GVHD	Viral Infection (day of onset)	Survival (months)	Current Status
1	AML (M5b)	22/M	0	—	—	22+	CR
2	AML (M2)	38/M	0	—	—	20+	CNS RLPS
3	ALL (L1)	16/F	I	—	VZV (47)	31+	CR
4	ALL (L1)	18/F	I	Localized	CMV (57), CMVIP (197)	27+	CR
5	ALL (L2)	18/M	0	Localized	—	30+	CR
6	SAA	39/F	I	Systemic	CMV (53), VZV (59), HSV (420)	40+	CR
7	SAA	43/M	0	Systemic	VZV (76)	17+	CR

FAB = the French-American-British classification of acute leukemias; AML = acute myelogenous leukemia; ALL = acute lymphoblastic leukemia; SAA = severe aplastic anemia; GVHD = graft-*versus*-host disease; VZV = varicella-zoster virus; CMV = cytomegalovirus; IP = interstitial pneumonitis; CR = complete remission; CNS RLPS = relapse in central nervous system.

cubation was followed by a 15-minutes wash in three changes of PBS. The cells were exposed to an alkaline phosphatase substrate solution consisting of naphthol-AS-B1-phosphoric acid as substrate, hexazotized new fuchsin as coupler, and levamisole for 10–20 minutes. Smears then were washed in PBS and counterstained with Mayer's hematoxylin.

Antibodies

The following purified murine monoclonal antibodies were used: anti-Ia-like antigen (I2), anti-CD10 (J5), anti-CD19 (B4), anti-CD20 (B1), anti-CD3 (T6), anti-CD3 (T3), anti-CD5 (T1), anti-CD13 (My7), anti-CD33 (My9), NKH-1, MsIgG as a negative control serum (Coulter Immunology, Hialeah, FL), antikappa (Yamasa Shoyu Company, Ltd., Tokyo, Japan), antilambda (BioGenex), antihuman mu heavy chain (antihuman IgM) (DAKO-PATTS A/S, Copenhagen, Denmark), and mouse anti-bovine TdT (received in kit form from Coulter).

RESULTS

On May-Grünwald-Giemsa–stained bone marrow smears, small blastoid cells were characterized by scant cytoplasm and dense nuclei with smooth homogeneous chromatin and ranged from 8 to 18 μm (median, 10.6 μm) in diameter (Fig. 1). Generally, there were no nucleoli. The cytoplasm was devoid of granules, inclusions, or vacuoles. These cells often exhibited distinct nuclear clefts.

In each case, the peak percentage of small blastoid cells ranged from 4.0 to 21.3% (median, 15%) and occurred between days 55 and 365 after transplant. The proportion of these cells decreased subsequently, at a rate that varied among patients.

There were no small blastoid cells in the peripheral blood of any patients. These cells showed no evidence of reactivity with PAS, alpha-naphtyl butylate esterase, naphthol AS-D chloroacetate esterase, or peroxidase stains. In six patients, immunocytochemical analysis of bone marrows with increased percentages of small blastoid cells revealed that these cells expressed TdT in 86–96% (median, 96%), Ia-like antigen in 26–42% (median, 40%), CD19 in 14–22% (median, 18%), CD10 in 26–50% (median, 30%), and cytoplasmic mu in 6–20% (median, 12%) of cases. No staining for CD20, immunoglobulin kappa or lambda light chains, CD1, CD3, CD5, NKH-1, CD33, and CD13 was found (Table 2).

Marrow engraftment occurred in all patients with 0.5
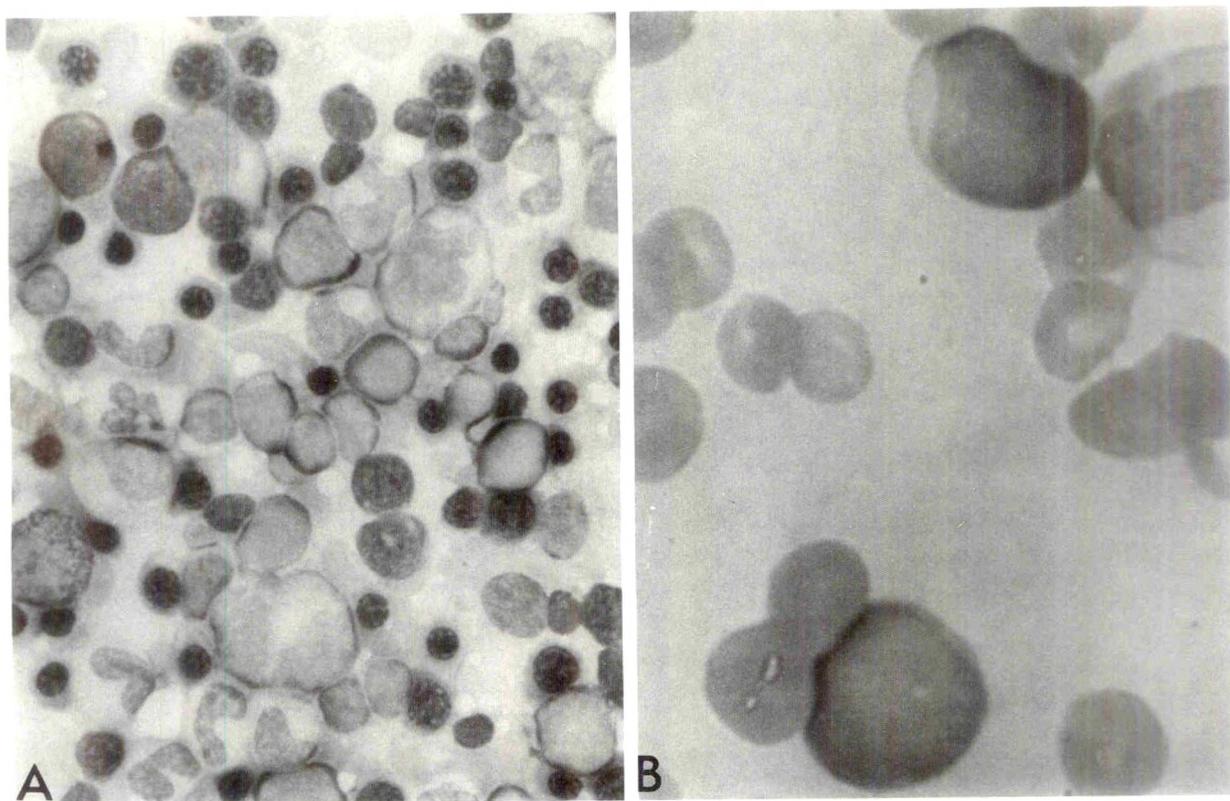
FIG. 1. Photomicrographs of May-Grünwald-Giemsa–stained bone marrow smears illustrate a spectrum of morphology of small blastoid cells. Small blastoid cells are characterized by scant cytoplasm and smooth homogenous chromatin. Nucleoli are absent or indistinct. *A (left)*, ×400. Small blastoid cells often exhibit nuclear clefts. *B (right)*, ×1,000.

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TABLE 2. IMMUNOCYTOCHEMICAL ANALYSIS OF SMALL BLASTOID CELLS

Patient No.	Day after BMT	% Small Blastoid Cells in Marrow	Diameter Mean (range) (μm)	Immunophenotype (%)													
				TdT	Ia	CD19	CD10	Cytoplasmic μ	CD20	κ Light Chain	λ Light Chain	CD1	CD3	CD5	NKH-1	CD33	CD13
1	55	14.5	10.7 (8–17)	86	30	18	26	6	0	0	0	0	0	0	0	0	0
	188	8.0	10.9 (8–17)	96	40	16	50	10	0	0	0	0	0	0	0	0	0
2	97	17.5	10.8 (8–18)	94	26	14	26	16	0	0	0	0	0	0	0	0	0
	183	19.0	11.0 (8–18)	96	36	14	30	18	0	0	0	0	0	0	0	0	0
3	98	21.3	10.0 (8–15)	96	40	16	48	16	0	0	0	0	0	0	0	0	0
	181	18.0	10.3 (8–15)	96	42	20	44	10	0	0	0	0	0	0	0	0	0
4	365	15.3	10.5 (8–17)	90	40	20	30	12	0	0	0	0	0	0	0	0	0
5	64	13.8	10.8 (8–18)	96	36	18	28	8	0	0	0	0	0	0	0	0	0
6	154	31.0	10.2 (8–15)	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
7	97	4.0	10.8 (8–15)	NT	40	22	36	20	0	0	0	NT	NT	0	0	0	0

NT = not tested.

× 10^9 granulocytes/L at a median of 27 days (range, 17–31 days) after transplant. Acute GVHD developed in two of the five allogeneic marrow recipients. Chronic GVHD occurred in three of these patients. Cytomegalovirus (CMV) infection/reactivation, although clinically causing no symptoms, was documented on day 57 after grafting in one of the six patients with small blastoid cells (patient 4); however, CMV pneumonitis developed by day 197. Other viral infections occurred in two patients (varicella zoster virus). A transient pancytopenia that probably resulted from viral infection (case 4) or some unidentified cause (case 1) preceded the appearance of small blastoid cells in the bone marrow. No clear temporal correlation was found between the time that small blastoid cells were observed and the onset of acute, chronic GVHD or viral infection. Follow-up ranged from 17 to 40 months. Five of six patients have shown no evidence of relapse. One patient (case 2) had a central nervous system relapse on day 395, but to date there has been no confirmation of bone marrow relapse.

DISCUSSION

This report describes the occurrence of a significant number of small blastoid cells in the bone marrow of marrow transplant recipients. Our studies of these cells confirmed that they were immature B-cell precursors. Our data on the expression of cytoplasmic mu and CD20 differed from those of other investigators,[11,12,18,19] who have described several distinct stages of B-cell maturation. For instance, Nadler and associates[18] investigated the expression of Ia, CD19(B4), CD10 (CALLA), and CD20(B1) antigens in 67 cases of non-T ALL using indirect immunofluorescence staining and flow cytometric analysis. These cases were subdivided into two groups, based on the presence or absence of cytoplasmic mu. These authors

concluded that the cytoplasmic mu–positive cells were more mature than CD20-positive S-precursor cells. In another study, Ryan and associates[20] reported that CD20 expression was unrelated to the presence of cytoplasmic mu in common ALL cells, in contrast to the expected pattern of normal CALLA-positive bone marrow lymphoid cells, wherein CD20 positivity is correlated strongly with cytoplasmic mu expression. Although the percentage of cytoplasmic mu–positive cells among all small blastoid cells was low in our study, we showed that cytoplasmic mu could be detected in the absence of CD20. The discrepancy between our results and those in earlier reports may result from the sensitivity of the ABC technique and its attendant amplification of target labeling.[21] However, our observation must be confirmed nonetheless by other investigators using both similar and disparate techniques.

Many investigators have characterized immature lymphoid, blastoid-appearing cells in bone marrow. Depending on the patient's age when the studies were performed, the methods used, and the hematologic disorders investigated, a variety of other terms have been applied to describe these cells, including hematogones,[2] posttherapeutic lymphoid cells,[3] precursor cells,[5] TdT-positive cells,[7,8] and CALLA-positive cells.[9-12] Although the parameters that have been studied only partially overlap among these reports, the cells are generally compatible with each other in terms of morphologic characteristics, cytochemistry, and immunophenotype and correspond to the small blastoid cells that we describe. Large numbers of morphologically similar cells have been documented in a variety of hematologic disorders, including infectious mononucleosis, hemolytic anemia, aplastic anemia, iron deficiency anemia, congenital neutropenia, idiopathic thrombocytopenic purpura, erythrogenesis imperfecta, and amegakaryocytosis; in children with retinoblastoma, neuroblastoma, rhabdomyosarcoma, ALL in remission, and BMT;

and in normal people.[2,5,8,22,23] Many patients described in the literature are young children—particularly newborn—or are those with actively regenerating marrows after a variety of cytopenias. We recognized an increased number of these cells in the marrows of several patients after bone marrow transplantation.

In two of the three patients with cytopenias during recovery from viral infection or resulting from an unknown cause within four months of BMT engraftment, an increased proportion of small blastoid cells was observed. Activated B cells putatively produce molecules capable of stimulating hemopoietic cells.[24-26] Perhaps the small blastoid cells participate in the regulation of hematopoiesis, not early after BMT, but later in the engrafted marrow.

Because these cells were identified as pre-B cells, their proliferation in the engrafted marrow may represent an immune response that is induced by antigenic stimulation, such as GVHD and viral infections. However, the emergence of increased numbers of small blastoid cells was observed in the absence of either GVHD (including one case of syngeneic BMT) or documented viral infections during the first year after transplant. Alternatively, increased numbers of small blastoid cells may result from hematopoiesis and immunologic reconstitution from stem cells after bone marrow transplantation. Hence, their presence may be regarded as part of a recapitulation of the ontogeny of the immune system. Nevertheless, the significance and function of these cells in immune recovery and hematopoiesis remain uncertain.

Because the morphologic, cytochemical, and immunophenotypic characteristics of small blastoid cells did not distinguish them reliably from leukemic lymphoblastic cells, especially in cases of FAB-L1 leukemia, these cells should be further evaluated in bone marrow of patients with ALL, especially L1, in remission. These cells exhibited a transient and self-limited increase in the marrow, whereas the increase of neoplastic blasts usually is a progressive one. It is important to remember that the occurrence of these small blastoid cells after BMT does not necessarily denote relapse.

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A Case of Acquired Factor X Deficiency with *In Vivo* and *In Vitro* Evidence of Inhibitor Activity Directed Against Factor X

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A 67-year-old woman had symptoms of an upper respiratory tract infection for which she received a five-day course of erythromycin. Epistaxis and gross hematuria subsequently developed, and the patient was found to have a selective Factor X deficiency. She received supportive therapy and prothrombin complex concentrates (Factors II, VII, IX, and X), with subsequent resolution of her transient Factor X deficiency. Her hospital course, however, was complicated by the development of multiple cerebral infarctions. This is the tenth reported case of transient Factor X deficiency not associated with amyloidosis. In seven of the previous cases, as in this patient, the deficiency was associated with a preceding upper respiratory infection. This is the only case, however, with evidence of inhibitory activity in the plasma that was directed toward Factor X. (Key words: Factor X inhibitor; Hemostasis; Hemorrhage; Prothrombin concentrate) Am J Clin Pathol 1991;96:196–200

Although acquired Factor X deficiency has been reported rarely in patients without amyloidosis, it is a well-described entity in patients *with* amyloidosis. The deficiency in these patients is attributed to enhanced clearance of circulating Factor X, resulting from the affinity of Factor X for amyloid fibrils.[1,2]

In 1959, Graham and associates described a case of transient Factor X deficiency in a patient after exposure to the fungicide methylbromide.[3] In 1966, Stefanini and Wiggishoff reported mild transient Factor X deficiency in a patient with renal and adrenal cortical carcinomas.[4] This mild deficiency resolved after the tumors were removed. It is interesting that the neoplasms were found incidentally while the patient was being evaluated for an upper respiratory tract infection. In 1969, Bayer and associates reported the case of a four-year-old boy with transient Factor X deficiency.[5] No infectious, malignant, or toxic associations were present in this case. In 1979, Peuscher and associates described a case of acquired Factor X deficiency

in a 57-year-old patient with lobar Mycoplasma pneumonia.[6] Five subsequent reports have described acquired Factor X deficiencies in patients with preceding upper respiratory tract infections.[7–11] In two cases, erythromycin had been administered.[8,11]

We report the case of a patient with transient Factor X deficiency that developed after a viral upper respiratory illness that was treated with erythromycin. Serologic studies for *Mycoplasma pneumoniae* were negative. This case is unique in that both *in vitro* and *in vivo* coagulation studies showed evidence of inhibitory activity in the plasma, directed toward Factor X. The patient was treated with prothrombin complex concentrates (PCCs), and multiple cerebral infarctions developed subsequently. The cause of these infarctions is unclear, but they may have resulted from a transient hypercoagulable state induced by factor-replacement therapy.

REPORT OF A CASE

This 67-year-old woman was admitted with epistaxis and gross hematuria. Her medical and family histories were unremarkable. Approximately one week before admission, she had fatigue, cough, fever, and chills develop after she swam in her pond. Her local physician examined her, and erythromycin and aspirin were prescribed empirically.

Severe epistaxis and gross hematuria developed subsequently, and she was admitted to her local hospital, where she received vitamin K and fresh-frozen plasma. She was transferred to the Medical Center Hospital of Vermont two days later, with continued brisk hemorrhage. Physical examination on admission showed stable vital signs, with a respiratory

rate of 32 breaths/minute. She had evidence of epistaxis and buccal mucosal bleeding. Diffuse rales and rhonchi were present throughout both lung fields. She also had frank melena and heme-positive stool. The remainder of the physical examination was unremarkable. Relevant admission laboratory data included a platelet count of 224 × 10⁹/L (normal range, 156–312 × 10⁹/L); prothrombin time (PT), 43.3 seconds (normal range, 11–13 seconds); activated partial thromboplastin time (APTT), 94 seconds (normal range, 25–37 seconds); thrombin time, 14.7 seconds (normal range, 12–20 seconds); bleeding time greater than 15 minutes (normal, 3–10 minutes); fibrinogen, 4.31 g/L (431 mg/dL) (normal range, 1.70–4.10 g/L [170–410 mg/dL]); and Factor X concentration, 4% (normal, 60–140%). The Factor II, V, and VII concentrations were within normal limits.

Hypoxia developed rapidly in association with diffuse bilateral fluffy infiltrates seen on chest roentgenogram, necessitating elective intubation. On the second hospital day, she was given PCCs (Profilnine® [Alpha Therapeutics; Los Angeles, CA], Hyland IX Complex® [Hyland Therapeutics; Glendale, CA]; Factor II, VII, IX, and X replacement) and prednisone, 60 mg intravenously, twice a day. After initial *in vivo* recovery studies with Factor X assays, the patient's response to therapy was monitored by APTT and her clinical bleeding status.

Over the next few days, her condition improved, with resolution of her bleeding and gradual correction of coagulation abnormalities (Fig. 1). Serologic studies were performed, which showed no evidence of recent adenovirus, parainfluenza, influenza A or B, Mycoplasma, respiratory synctial virus (RSV), herpes, cytomegalovirus, or Epstein-Barr virus infections. She had negative Legionella titers and a normal serum electrophoresis. The patient was extubated on hospital day 12, at which time her factor-replacement therapy was discontinued.

Two to three days after cessation of PCC administration, the patient had right leg weakness, right body dyspraxia, and a deterioration in mental status. A computed tomography (CT) scan of her head at this time was unremarkable. The patient's neurologic status improved over the next several days; however, she still had abnormal mentation, with flight of speech, labile affect, and persistent right leg apraxia. A repeat CT scan

FIG. 1. Changes in hemostatic parameters over the hospital course are shown diagramatically. Factor X (▲——▲) and prothrombin (●--●) levels are expressed as percent activity (1 μ/mL pooled normal plasma = 100%) on the left X axis. The activated partial thromboplastin time (▣···▣) is expressed in seconds in the right y-axis. The *inset* figure in the right upper quadrant is an expanded view of the data from the box in the left lower quadrant demonstrating the disappearance time of Factor X following the initial prothrombin concentrate infusion Factor X (△——△).

on the twentieth hospital day showed multiple cerebral infarctions; some of which were in the "watershed" distribution. These lesions were consistent with an embolic or ischemic event. She was discharged subsequently to a rehabilitation facility and has recovered partially.

MATERIALS AND METHODS

Factor VII–deficient substrates were purchased frozen from the New England Regional Hemophilia Center (Worcester, MA). Factor II and X deficient substrates were lyophilized plasma purchased from Dade (Miami, FL). Factor V–deficient plasma was prepared by immunodepletion as previously described.[12] Lyophilized pooled plasma for 50/50 mixing studies was purchased from Dade (Miami, FL). Purified human Factor X was prepared by the method of Bajaj and colleagues[13] and activated to Factor Xa with purified Factor X activator from Russell's viper venom (RVV-X), as described previously.[14] RVV-X was isolated as described by Kisiel and colleagues.[15] Prothrombin was purified from citrated frozen plasma as previously described.[13]

Plasma Collection and Processing

Blood for PT, partial thromboplastin time (PTT), fibrinogen, thrombin time, and factor assays was collected in 0.1 mol/L sodium citrate–citric acid at a 9:1 whole blood–anticoagulant ratio. Plasma for platelet counts was collected in ethylenediaminotetraacetate (7.5 mg) Vacutainer® tubes (Becton Dickinson, Rutherford. NJ). Blood samples for fibrin degradation product analyses were collected in tubes containing thrombin (20 National Institutes of Health (NIH) units per tube) and soybean trypsin inhibitor (approximately 3,600 NF units per tube) (Thrombo-Wellcotest® Collection Tube; Burroughs Wellcome Company, Research Triangle Park, NC).

Factor Assays and Screening Tests

Prothrombin time and APTT were performed by standard automated methods using Dade Thromboplastin C · reagent (Dade, Aguada, Puerto Rico) and General Diagnostics Auto APTT® reagent (General Diagnostics, Morris Plains, NJ). Functional fibrinogen levels were assessed with a Dade Fibrinogen Kit® (Dade, Miami, FL) on a BBL Fibrosystem® fibrometer, according to the manufacturer's instructions. Fibrin degradation products were measured with the use of the Thrombo-Wellcotest (Burroughs-Wellcome, Dartford, UK). Standard Ivy bleeding times were performed with the use of the General Diagnostics Simplate II® device. Our normal range, based on a sample of 100 evenly matched male and female subjects, is 3–10 minutes. Thrombin times were obtained by adding bovine thrombin (10,000 NIH units; Parke-Davis, Morris Plains, NJ) to citrated plasma. The thrombin had

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been adjusted to give a clotting time of 15.5–16.5 seconds in normal plasma. Platelet counts were performed on a Coulter S+IV® by standard methods.

Factor II and X coagulation assays were measured by a one-stage tilt-tube method against pooled reference plasma, as previously described.[16] Mixing studies were performed by adding patient plasma to an equal quantity of normal plasma and measuring the PT and APTT as described above. Total Factor V activity was measured by a two-stage assay as described previously.[16]

Two-stage Factor Xa assays were performed by activation of plasma with Russell Viper Venom (Wellcome Reagents, Ltd, Dartford, UK) before assay. Patient plasma, normal plasma, and an equal mix of patient and normal plasma were assayed.

Purified prothrombin was added to normal sodium citrate–anticoagulated plasma to increase the prothrombin concentration from 100 mg/L (100 μg/mL) to 500 mg/L (500 μg/mL). PTs and PTTs were performed on these plasmas and appropriate control plasmas to evaluate the effect of high prothrombin concentrations on the screening assays.

RESULTS

In view of the patient's prolonged PT and PTT on admission, 50/50 mixing studies were performed, and, as illustrated in Table 1, these showed only partial correction. This finding suggested the presence of an inhibitor rather than an isolated factor deficiency.

To determine the specificity of the inhibitor, extrinsic and common pathway factor assays (Factors II, VII, V, and X) were performed. Factors II, VII, and V were normal; Factor X was 4%.

A Factor X assay using RVV-X activator was performed on an equal mixture of patient and normal plasma using a Russell's Viper Venom Factor X assay. A Factor X level of 32% was observed in the equal part mixture, in contrast to the 50% Factor X level observed in the control plasma. This inhibitory activity was verified by addition of purified human Factor Xa to patient plasma and normal plasma (10 mg/L [10 μg/mL], final concentration). After a 5-minute incubation, there was significant prolongation of the clotting time in the patient plasma compared with the normal pooled plasma (Table 2).

TABLE 1. MIXING STUDIES

PT (seconds)			APTT (seconds)		
Normal	Patient	1:1 Mix	Normal	Patient	1:1 Mix
12	43.3	14.2	31	94	37

PT = prothrombin time; APTT = activated partial thromboplastin time.

TABLE 2. EFFECT OF ADDITION OF PURIFIED FACTOR Xa ON RUSSELL VIPER VENOM CLOTTING ASSAY

Reaction Mixture	Incubation	
	Immediate	5 Minutes
F Xa + normal plasma	22.1 seconds	35 seconds
F Xa + patient plasma	24.7 seconds	65.6 seconds

F Xa = activated Factor X.

The Factor IX concentration is identified for commercially available Factor IX concentrates, but they also contain variable amounts of prothrombin, Factor X, and Factor VII. So that *in vivo* recovery studies could be performed more accurately, Factors IX and X and prothrombin were measured in several brands and lot numbers of factor concentrates used to treat this patient. The findings are shown in Table 3.

In vivo recovery studies, performed after infusion of PCCs early in the patient's hospital course, showed an accelerated clearance of Factor X. An estimated half-life of four hours was observed, which is considerably shorter than the expected half-life of 30 hours (Fig. 1).[12] In contrast, prothrombin levels increased rapidly to more than 400%. In addition, with clearance of the X inhibitor and continued concentrate therapy, the Factor X level increased to a peak of 345% on the tenth day of factor replacement (Fig. 1). The addition of large amounts of prothrombin (similar to the levels achieved in this patient) to normal plasma increased the PTT by 1.4 times and the PT by 1.3 times. The initially prolonged bleeding time was normal by the third day of concentrate therapy.

DISCUSSION

This is the tenth reported case of non–amyloidosis-associated transient Factor X deficiency (Table 4). In pre-

TABLE 3. ASSAYED LEVELS* OF FACTORS IX, X, AND II IN PROTHROMBIN COMPLEX CONCENTRATES

A. Alpha Therapeutics Profilnine Lot CW7007A
    (vial assay value = 570 μ F IX)
        Factor IX = 570 U/vial
        Factor X = 432 U/vial
        Factor II = 576 U/vial
B. Hyland IX Complex Lot 2836011A
    (vial assay value = 600 μ F IX)
        Factor IX = 648 U/vial
        Factor X = 548 U/vial
        Factor II = 606 U/vial
C. Hyland IX Complex Lot 2836010AA
    (vial assay value = 600 μ/F IX)
        Factor IX = 605 U/vial
        Factor X = 436 U/vial
        Factor II = 612 U/vial

F IX = Factor IX.
* Concentration designated "U" for units with one unit being the amount of the specific coagulation factor activity present in 1 mL of pooled normal plasma.

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TABLE 4. TRANSIENT FACTOR X DEFICIENCY

Investigator	Associated Disease	Transient	Inhibitor Demonstrated Directly	Antibiotics
Graham[3] and associates	Fungicide	Yes	No	—
Stefanini and Wiggishoff[4]	Tumor: acute pulmonary disease	Yes	No	—
Bayer and associates[5]	Nil	Yes	No	—
Peuscher and associates[6]	Acute pulmonary disease (mycoplasma)	Yes	No	Penicillin
Edgin and associates[9]	Acute pulmonary disease (viral)	Yes	No	—
Bayer and associates[7]	Acute pulmonary disease (viral)	Yes	No	Penicillin
Hosker and Jewell[11]	Acute pulmonary disease (viral)	Yes	No	Erythromycin
Currier and associates[8]	Acute pulmonary disease (viral)	Yes	No	Erythromycin
Henson and associates[10]	Acute pulmonary disease (viral)	Yes	No	—

vious case reports, inhibitory activity could not be documented in association with the factor deficiency. In this case, the existence of an inhibitor was demonstrated clearly by *in vivo* and *in vitro* evidence. *In vitro* studies established the presence of a specific inhibitor to Factor X and Xa. *In vivo* recovery studies after infusion of PCCs showed a significantly decreased Factor X half-life.

Seven previously reported cases of transient Factor X deficiencies also were preceded by upper respiratory tract infections.[4,6-11] It is hypothesized that antibodies to the microorganisms cross-reacted with the patient's Factor X. However, attempts to isolate specific immunoglobulins directed against Factor X in our patient were unsuccessful. Three patients, including this one, received erythromycin for their infections. It is not known whether this antibiotic played a role in the development of the factor deficiencies.[8,11]

The patient's clinical bleeding complications and laboratory hemostatic abnormalities improved dramatically after her factor concentrate replacement. It was believed that her acute respiratory failure resulted from intrapulmonary hemorrhage, which also responded to the replacement therapy. The initially prolonged bleeding time with a normal platelet count may have been related to her aspirin therapy. In addition, an abnormal bleeding time has been reported previously in a patient with acquired Factor X deficiency and associated defects in platelet aggregation.[9]

Another interesting feature in this case is the patient's subsequent development of multiple cerebral infarctions. The patient first manifested neurologic symptoms two to three days after factor replacement therapy was discontinued. There were no preceding hypotensive or ischemic events. On CT scan, these lesions were compatible with either embolic or ischemic infarctions with multiple, discrete, nonhemorrhagic lesions. The relationship of factor replacement therapy to the cerebrovascular events in this patient is unclear. It is conceivable that this treatment, which resulted in significant increases in the prothrombin and Factor X levels, predisposed her to the development of multiple thrombotic emboli. Thrombotic complications have been described in patients treated with PCCs.[17-19] The most serious of these include myocardial infarctions and disseminated intravascular coagulation. In a previously reported case, a large myocardial infarct developed in a 15-year-old hemophiliac, for which the patient subsequently received a cardiac transplant.[18] It is interesting that the Factor X level was 400% at the time of infarction.

Factor X replacement with PCCs appears to have been effective treatment for the acute bleeding episode. The patient's PT and PTT improved dramatically after replacement therapy was started but did not become completely normal until three days after PCC treatment was stopped. However, her bleeding clinical diathesis improved promptly, coincident with replacement therapy. Consequently, after the initial Factor X recovery studies, the patient's response to replacement therapy was monitored by the PT and APTT. Assays for Factors II and X that were performed subsequently demonstrated extremely high levels of prothrombin. The high levels of prothrombin appear to have affected the PT and PTT and may have played a role in the thromboembolic complication. The cause of the prolonged PT and PTT associated with high levels of prothrombin is not clear, but may have resulted from increased "self-association"[20] at these high concentrations. Such polymerization might hinder normal prothrombinase function. We conclude that PCCs constitute effective treatment for transient Factor X inhibitors.

However, Factor X levels should be measured to monitor therapy, to avoid unnecessarily high levels of vitamin K–dependent procoagulant proteins.

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Red Blood Cell Alloimmunization Complicating Plasma Transfusion

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Well-known adverse effects of plasma transfusion include viral transmission, allergic complications, and rare anaphylactic reactions. In making clinical decisions to transfuse plasma, a seldom-considered complication is that of red blood cell (RBC) alloimmunization. The authors report a patient in whom strong IgM and IgG anti-E and weak IgG anti-JK^a RBC antibodies developed 15 days after infusion of two units of fresh-frozen plasma for volume expansion. These antibodies are potentially

hemolytic. This case underscores the importance of considering risks of plasma infusion. Plasma should not be used casually, especially for indications for which alternate therapies, such as crystalloid and colloid solutions, are available. (Key words: Red blood cell alloimmunization; Fresh-frozen plasma; Plasma infusion indications; Plasma infusion complications) Am J Clin Pathol 1991;96:201–202

Although there is a consensus that definitive indications for the transfusion of plasma are limited,[1] and there is mounting evidence of the potential risk of blood product transfusion, plasma continues to be used in clinical settings where alternative and safer therapies are available. Among the well-known complications of plasma transfusions are viral disease transmission, allergic reactions, and rare anaphylactic reactions. Another rarely discussed complication is alloimmunization to red blood cell (RBC) antigens.[2] Alloantibodies to a number of RBC antigens have been associated with hemolytic transfusion reactions and hemolytic disease of the newborn.[3] We report the case of a patient in whom detectable antibodies developed against antigens that are generally considered to have low immunogenicity—E and JK^a—after transfusion of only two units of fresh-frozen plasma.

REPORT OF A CASE

This 71-year-old woman (para 0, gravida 0) was admitted for treatment of a bleeding intracranial aneurysm. Nine years earlier, she had received four units of RBC concentrate during a hip arthroplasty. On this ad-

mission, an initial antibody screen, done when two units of RBCs were requested, showed no detectable antibodies. These units were not given, but the patient received two units of fresh-frozen plasma as a volume expander. Fifteen days after the plasma infusion, cross-matching for RBC concentrates, as required for a craniotomy, revealed the presence of anti-E and anti-Jk^a.

MATERIALS AND METHODS

Reagents from commercial sources (Immucor Canada Ltd., Edmonton, Alberta, Canada) for the detection and identification of antibodies against RBC antigens were used according to the manufacturer's instructions. Pretransfusion testing was performed by standard techniques, using low ionic strength saline, antibody identification, phenotyping, dithiothreitol inhibition, and grading.[4]

RESULTS

The patient's RBC phenotype was A Rho(D) positive, and no unexpected antibodies were found on admission. Fifteen days after the fresh-frozen plasma transfusion, a strong IgM and IgG anti-E and a weak IgG anti-Jk^a were detected (see below). An autologous control, done to exclude autoantibodies, was negative. Four units of E−, Jk(a−b+) RBC concentrates were cross-matched successfully.

A 4+ RBC agglutinate was observed when the patient's serum was mixed and spun with panel RBCs for the identification of anti-E. This reaction could be abolished by pretreating the serum with 0.01 mol/L dithiothreitol, which suggested that the antibody was of the IgM type.

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The additional presence of IgG anti-E was detected by the indirect antiglobulin test using gamma chain–specific antihuman globulin. Appropriate controls were included to eliminate the possibility of agglutination resulting from IgM antibodies. The anti-Jk^a antibody could be demonstrated only by the indirect antiglobulin test when homozygous Jk(a+b−) panel RBCs were used in low ionic strength saline or when panel RBCs were pretreated with ficin.

The donors of the two units of fresh-frozen plasma had negative antibody screens. One was typed as A, Rho(D) positive, E−, Jk(a+b−). The second donor was typed as A, Rho(D) positive, E+, Jk(a+b−).

DISCUSSION

Red blood cell alloantibodies usually develop as a result of RBC transfusion or as a complication of pregnancy. Intact RBCs in liquid plasma (*i.e.*, plasma prepared from whole blood and stored at 4 °C for more than six hours) can stimulate RBC antibody production occasionally.[2] Plasma that is separated from whole blood within six hours of collection and snap frozen (fresh-frozen plasma) contains only traces of RBC stroma, which is cleared rapidly from the circulation after infusion, and causes immune response only rarely.[2,5] As little as 0.024 mL of Rh-positive RBCs, or perhaps less, can cause primary sensitization in Rh-negative people.[6] Conceivably, a much lower dose is required to induce an anamnestic response.

A strong anti-E of both IgM and IgG isotypes and a weak anti-Jk^a developed in our patient after transfusion of two units of fresh-frozen plasma. Both donors were Jk(a+b−), and one was E-positive. The E antigen is thought to be 30 times and the Jk^a antigen approximately 720 times less immunogenic than the D antigen.[7] It is possible that the two units of fresh-frozen plasma that were transfused were contaminated with a sizable amount of RBC stroma, even though they showed no hemolysis or RBC contamination on gross inspection. Although IgM RBC antibodies usually are considered to result from a primary immune response, we have no data to exclude the possibility of prior immunization to both the E and Jk^a antigens at the time of transfusion nine years previously. To our knowledge, the development of anti-E and anti-Jk^a after plasma transfusion has not been reported.

The National Institutes of Health consensus conference suggested that a tenfold increase in fresh-frozen plasma use had occurred in the past decade, although the indications for its use are limited.[1] The circumstances in which plasma is considered to be of therapeutic value include replacement of isolated coagulation factors, reversal of the effects of coumarin-type drugs, massive transfusion, antithrombin III deficiency, treatment of immunodeficiencies, and therapy of thrombotic thrombocytopenic purpura.

Despite ample evidence of considerable risk that is associated with blood product transfusion in clinical settings, as much as 90% of plasma is used for volume replacement or other reasons, including nutritional supplementation, which cannot be justified.[8] The risks of plasma transfusion are considered acceptable when alternative treatment is not available; however, in most cases it is evident that many patients can be given safer products, such as crystalloid or colloid solutions.[9] In addition to the recognized hazard of transmission of viral infection, including hepatitis and possibly human immunodeficiency virus, there is also the possibility of alloimmunization to RBCs (and, by extension, white blood cells)—as illustrated in our case—as well as plasma proteins.

Alloimmunization to RBCs seldom is considered in decisions to transfuse plasma products. The RBC antibodies detected in our patient are recognized to produce hemolytic transfusion reactions and hemolytic disease of the newborn in the proper clinical settings.

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Use of Fluorescent *In Situ* Hybridization for Marker Chromosome Identification in Congenital and Neoplastic Disorders

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Identifying marker chromosomes of unknown origin in the clinical cytogenetics laboratory has been a problem historically, despite advances in specialized staining techniques. Determination of the origin of these marker chromosomes in patients with congenital or malignant neoplastic disorders is essential for more complete diagnosis, counseling, and treatment. The authors describe the use of fluorescent *in situ* hybridization with chromosome-specific alpha-satellite DNA probes to identify the origin of marker chromosomes in two patients with congenital disorders and three patients with malignant neoplastic disorders. The impact of firm identification of the marker chromosome for the diagnosis of these patients is discussed. The authors also discuss the feasibility of using this technique routinely in the clinical cytogenetics laboratory. (Key words: *In situ* hybridization; Marker chromosome identification; Hematologic disorders; Congenital disorders) Am J Clin Pathol 1991;96:203–210

Significant advances have continued in chromosome analysis and identification since the advent of chromosome banding techniques. Despite this progress in specialized techniques such as silver stains for nucleolar-organizing regions (AgNOR), anticentromere stains, and distamycin/4' 6' Diamino-2'-phenyl-indole (DAPI) staining, there are still many congenital and hematologic cases exhibiting marker chromosomes that cannot be identified by these techniques. We describe the use of chromosome centromere-specific alpha-satellite DNA probes with fluorescent *in situ* hybridization for identification of marker chromosomes of unknown origin in congenital and neoplastic disorders.

MATERIALS AND METHODS

Chromosome preparations were obtained from phytohemagluttinin (PHA) stimulated peripheral blood lymphocyte cultures with the use of standard techniques[1] or from bone marrow direct or short-term unstimulated cultures harvested with robotic methods.[2] Metaphases were stained for G-bands by trypsin and Leishman's stain (GTL) using a modification of the Seabright procedure.[3] Q-bands by fluorescent quinacrine mustard (QFQ) using a modification of the method of Caspersson and colleagues,[4] and C-bands by barium hydroxide and Leishman's stain (CBL) using a modification of the method of Sumner.[5] Metaphase analysis followed guidelines of the Association of Cytogenetic Technologists (ACT).[6]

Fluorescent *in situ* hybridization was performed as follows: Biotin-labeled alpha-satellite DNA chromosome-specific probes (Oncor, Gaithersburg, MD), at 1.5–2.0 ng/ μL in a hybridization mix containing 500 μg/mL sonicated herring sperm DNA, 50% formamide, 2 × SSC (300 mmol/L sodium chloride, 30 mmol/L sodium citrate) and 10% dextran sulfate, were applied to chromosome preparations, coverslipped, and sealed with rubber cement Chromosomal and probe DNA were denatured for 10 minutes in an 80 °C oven; then hybridization was allowed to take place overnight (up to 20 hours) at 37 °C in a moist chamber. Posthybridization washes and fluorescent signal amplification steps were performed according to the methods of Pinkel and associates.[7] The conditions of posthybridization wash stringency were determined with the use of information provided with the probes. Chro-

mosomes were counterstained with 0.25 μg/mL propidium iodide (PI) in the antifade compound 1,4 diazabicyclo(2,2,2)octane. Fluorescent *in situ* hybridization was performed on unstained slide preparations that had not been previously analyzed cytogenetically.

Hybridization sites were indicated by a yellow fluorescent signal on the red fluorescent counterstained chromosomes upon excitation at 390–490 nm (filter H2; Leitz, Cincinnati, OH). Marker chromosomes were identified within the metaphases by a trained cytogenetic technologist (C.R.S. or W.J.K.), and the presence or absence of centromeric fluorescence was noted. Signal intensity was compared with the normal homolog within each metaphase. Kodak Ektachrome 400® (Eastman Kodak, Rochester, NY) film was used to photograph metaphase cells.

Before use on patient samples, each probe was evaluated on normal control metaphase preparations. For each probe used, more than 80% of the control metaphases contained two easily identifiable sites of centromeric fluorescence on an appropriately sized chromosome with the correct centromere index. Less than 1% of the control metaphases contained three hybridization signals.

In some instances, the presence or absence of Y-DNA was confirmed by Southern blot analysis with the use of hybridization protocols described by Thibodeau and associates.[8] Most Y-DNA probes were purchased from the American Type Culture Collection (ATCC, Rockville, MD).

RESULTS

Patient 1

This four-year-old girl appeared to be of normal intelligence, although formal development assessment was not done; however, she was relatively short in stature (5th percentile). Other Turner features also were observed, so cytogenetic analysis was requested to establish the patient's karyotype. Analysis of 30 cells from a PHA-stimulated lymphocyte culture revealed a karyotype of mos45,X/46,X,?r(?) (Fig. 1A). Two cells had a 45,X karyotype, and 28 cells had one normal X chromosome and a small marker chromosome that resembled a ring chromosome. Fluorescent *in situ* hybridization with alpha-satellite DNA probes specific for chromosomes X[9] and Y[10] revealed hybridization-specific fluorescence on the marker for only the X chromosome probe (Fig. 1B). Southern blot analysis of several regions of the Yq and Yp arms using probes 47Z,[11] p50f2,[11] pDP34,[12] Y97,[13] p12f,[11] pY3.4,[14] and pDP1007[15] excluded the presence of other Y-specific sequences within the patient's DNA (data not shown).

Patient 2

In 1981, at age 11, this patient had features of Turner's syndrome and was referred for cytogenetic analysis at another institution. Analysis revealed a karyotype of mos45,X/46,X,+mar/46,X,+frag/47,X,+mar,+frag. Fourteen cells had a 45,X karyotype, 5 cells had a normal X and a marker chromosome, 13 cells had a normal X and a small fragment, and 14 cells had a normal X and both the marker and the fragment (ratio 3:1:3:3). With estrogen therapy, she underwent normal pubertal development. She now has normal menses and is attending college.

Chromosome analysis performed at a second institution when the patient was 21 years old indicated that the same four cell lines were present, but in a 1:3:3:9 ratio (125 metaphases analyzed). Additional analysis showed the marker chromosome to be C-band positive at one end. These results were duplicated by our laboratory with cells provided by the second laboratory (Figs. 2A and B). Fluorescent *in situ* hybridization with the X- and Y-specific alpha-satellite probes showed both the fragment and marker chromosomes to be derived at least partially Y chromatin (Fig. 2C). The marker chromosome exhibits fluorescence at two distinct sites, even though only a single C-band was observed. Hybridization results therefore indicate the presence of Y-centromeric DNA at three different sites among the two marker chromosomes. The presence of other Y-DNA sequences detected by probes pDP34, pDP1007, 47Z, p50f2, p12f, and Y97 was confirmed by Southern blot analysis, but Y-DNA detected by probe pY3.4 was not present in total patient DNA (data not shown).

Patient 3

At age 19 years, this man presented with a primary mediastinal germ cell tumor. Two courses of bleomycin, etoposide, and cisplatinum chemotherapy were followed by surgical resection in April 1988. Two additional courses of bleomycin, etoposide, and cisplatinum were completed in June 1988. On a routine follow-up visit in March 1989, he had modest leukopenia (white blood cell [WBC] count, 3.8 × 10⁹ cells/L) with 2% blasts in the differential count. A bone marrow aspirate performed in May 1989 revealed 16% blasts, with marker studies showing the cells to be terminal deoxynucleotidyl transferase (TdT) negative. At that time, chromosome analysis performed at another institution indicated two apparently unrelated clones. One clone contained monosomy 7, with an unbalanced derivative chromosome 5 [(5)t(?3;5)(q21;q22)], an apparently balanced t(14;18)(q11;p11.2), and one marker chromosome. The second clone contained monosomy 5 and two
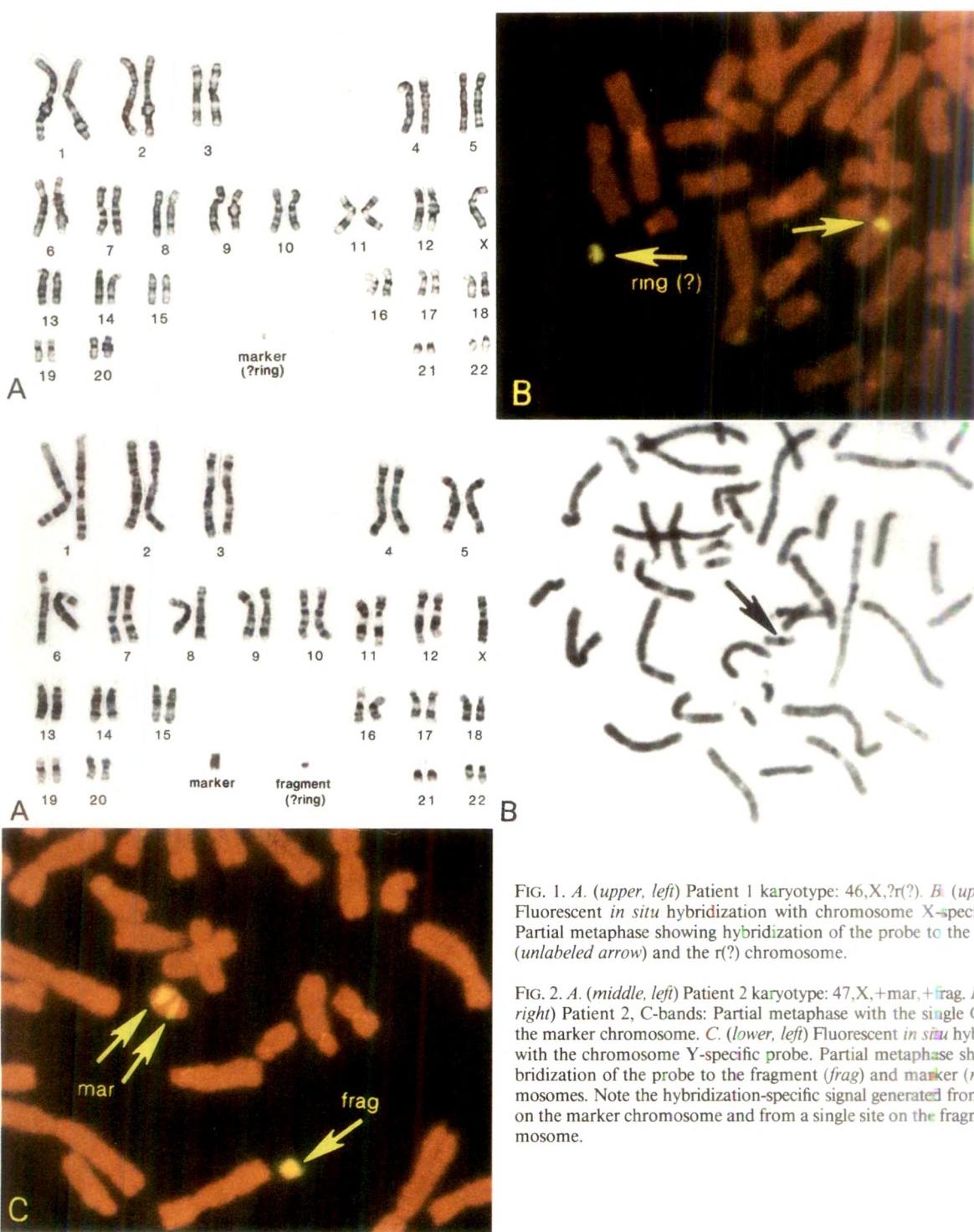
FIG. 1. *A.* (*upper, left*) Patient 1 karyotype: 46,X,?r(?). *B.* (*upper, right*) Fluorescent *in situ* hybridization with chromosome X-specific probe. Partial metaphase showing hybridization of the probe to the normal X (*unlabeled arrow*) and the r(?) chromosome.

FIG. 2. *A.* (*middle, left*) Patient 2 karyotype: 47,X,+mar,+frag. *B.* (*middle, right*) Patient 2, C-bands: Partial metaphase with the single C-band on the marker chromosome. *C.* (*lower, left*) Fluorescent *in situ* hybridization with the chromosome Y-specific probe. Partial metaphase showing hybridization of the probe to the fragment (*frag*) and marker (*mar*) chromosomes. Note the hybridization-specific signal generated from two sites on the marker chromosome and from a single site on the fragment chromosome.

different marker chromosomes. One of these two marker chromosomes had the general appearance of an isochromosome 12p, although this identification was uncertain. Acute nonlymphocytic leukemia (ANLL) was diagnosed, and induction chemotherapy with high-dose cytosine arabinoside and daunorubicin was administered. Subsequent chromosome analysis of bone marrow aspirate, performed at the same institution, indicated the continued presence of the second cell line despite evidence of morphologic remission.

The Mayo Clinic Cytogenetics Laboratory performed chromosome studies on a direct bone marrow preparation

just before a bone marrow transplant in early March 1990. The patient's father was the donor. This analysis revealed a karyotype of 46,XY=5/47,XY,+mar=5 (Fig. 3*A*). The marker chromosome appeared similar to an isochromosome 12p, but positive identification was not possible. Four weeks after the transplant, a follow-up bone marrow aspirate was performed; the findings were consistent with early regeneration. Cytogenetic analysis revealed a karyotype of 46,XY=2/47,XY,+mar=3/47,XY,inv(5) (q22q23),+mar=15. The marker chromosome of this analysis again appeared similar to an isochromosome 12p. Fluorescent *in situ* hybridization with a chromosome 12–specific alpha-satellite DNA probe (provided by Dr. Huntington Willard, Stanford University) revealed the marker to be brightly fluorescent (Fig. 3*B*). Both normal chromosome 12 centromeres were brightly fluorescent. No other normal chromosomes within the metaphases were positive. The fluorescence of the marker was approximately twice that of the normal chromosomes 12 in the same metaphases. This suggests that the marker contains two complements of chromosome 12 alpha-satellite sequences. Although these data strongly suggest that the marker is an i(12p), we cannot exclude the presence of DNA sequences from other chromosomes within it.

Shortly after the last cytogenetic analysis, severe graft-*versus*-host disease developed and the patient died in April 1990.

Patient 4

This 64-year-old man presented in April 1990, with a two-month history of weakness and fatigue. A peripheral blood smear was morphologically consistent with macrocytic normochromic anemia (hemoglobin [Hb], 9 g/dL; WBC count, 4.7 × 10⁹ cells/L; platelet [Plt] count, 126 × 10⁹/L). The bone marrow was hypercellular, with granulocytic hyperplasia, and contained 3% blasts. Chromosome studies were not performed.

Myelodysplastic syndrome (MDS) was diagnosed, and treatment with folic acid and pyridoxine was started. Weakness and fatigue persisted, and a peripheral smear performed in May 1990 contained rare circulating blasts. A second bone marrow aspirate was performed, and granulocytic hyperplasia was observed with increased immaturity of the granulocytic cells. Scant fat was present. Blast cells constituted 3.9% of the differential count. Cytogenetic analysis of 20 metaphases from direct bone marrow preparations indicated a karyotype of 46,XY=3/46,XY,−7, +r(?)=17 (Fig. 4*A*). Additional analysis showed the r(?) to be C-band positive (Fig. 4*B*). Fluorescent *in situ* analysis with a chromosome 7–specific alpha-satellite DNA probe[16] indicated that the r(?) was of chromosome 7 origin (Fig. 4*C*). In each of five normal metaphases examined that were from the direct bone marrow preparation, both

chromosome 7 centromeres exhibited fluorescence. In each of ten abnormal metaphases examined, both the normal chromosome 7 and the marker chromosome revealed bright centromeric fluorescence. No other chromosome within the metaphases was positive. The fluorescence of the marker chromosome indicates the presence of chromosome 7 alpha-satellite DNA sequences. We cannot exclude the presence of other chromosomal DNA sequences within the marker.

Within two months of the second bone marrow aspirate, the patient was found to have 12% blasts in the peripheral blood. Approximately 30% blasts and atypical monocytosis were observed in a third bone marrow specimen. The blasts were peroxidase positive and contained Auer rods. Rapidly progressive acute nonlymphocytic leukemia was diagnosed (French–American–British classification M2). The patient was treated with aggressive chemotherapy (daunomycin, cytosine arabinoside, 6-thioguanine, and prednisone) and entered a partial remission. Four months later (seven months after initial presentation), the patient died of progressive leukemia. An autopsy was not performed.

Patient 5

This 71-year-old man presented in April 1990, with a three-month history of bruising, epistaxis, and mild angina upon exertion. His peripheral blood counts and peripheral smear were consistent with anemia and thrombocytopenia. The bone marrow was hypercellular, with dyserythropoiesis and dysmegakaryocytopoiesis. Cytogenetic studies were not performed.

Danazol treatment was started, and the patient's platelet count increased. Within one month, his epistaxis returned and low-grade fever with night sweats developed. Again, he was found to be anemic and thrombocytopenic (WBC count, 4.1 × 10⁹ cells/L; Hb, 8.8 g/dL; Plt count, 18 × 10⁹ cells/L; peripheral blood contained 23% blasts). A repeat bone marrow aspirate was significantly hypercellular, with 75% blasts. Special stains were performed and consistent with acute lymphocytic leukemia (ALL) (TdT and common acute lymphoblastic leukemia antigen [CD10]—positive; peroxidase, butyrate esterase, and chloroacetate esterase—negative). Cytogenetic analysis of 20 metaphases from both a direct preparation and a 24-hour culture without mitogens revealed a karyotype of 46,XY,−20, + mar (Fig. 5*A*). Fluorescent *in situ* hybridization with a chromosome 20–specific alpha-satellite DNA probe[10] revealed fluorescence for the normal chromosome 20, as well as the marker chromosome (Fig. 5*B*), in each of seven metaphases examined. No other normal chromosomes within the metaphases showed significant fluorescence. This indicates that the marker contained chromosome 20
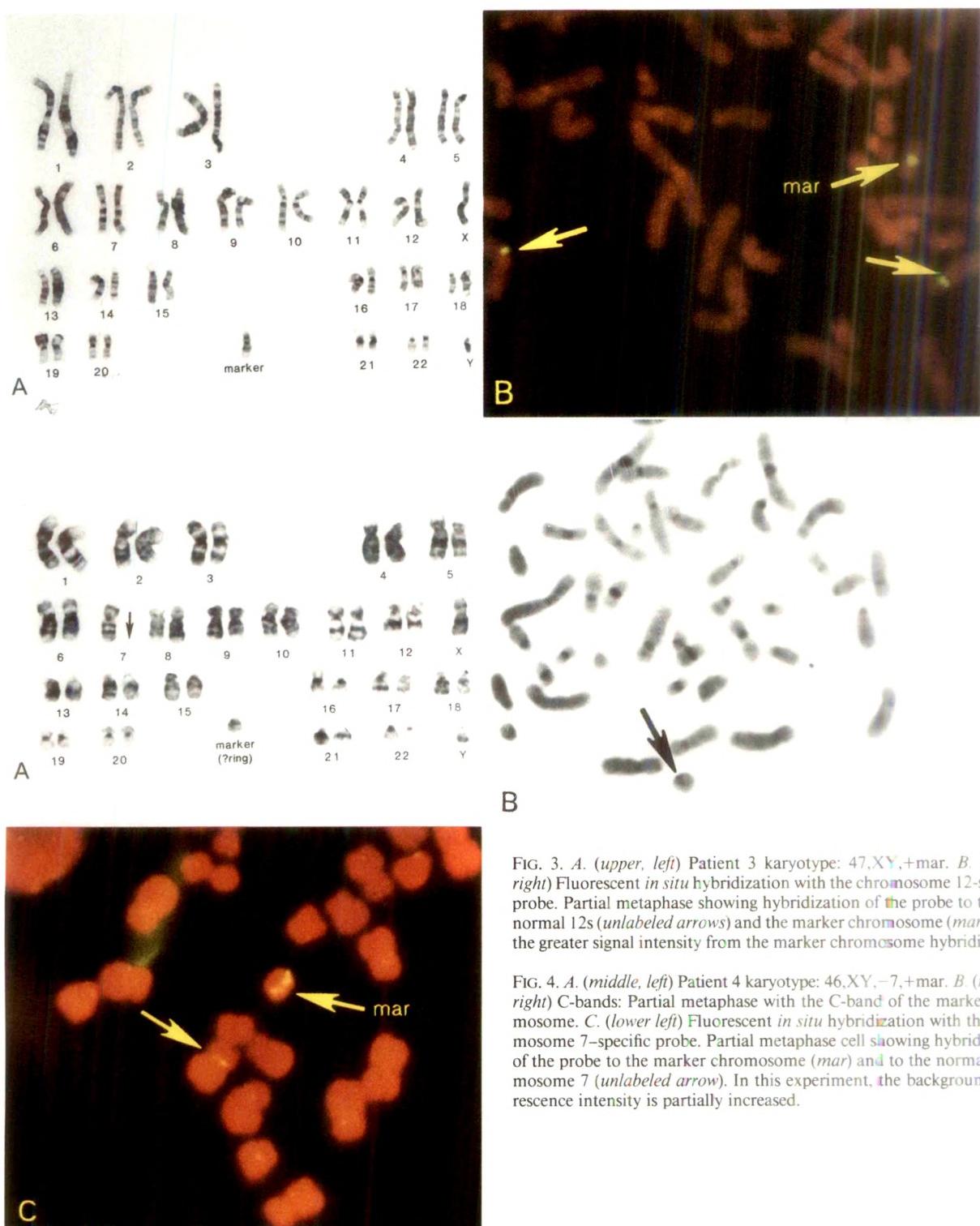
FIG. 3. *A. (upper, left)* Patient 3 karyotype: 47,XY,+mar. *B. (upper, right)* Fluorescent *in situ* hybridization with the chromosome 12-specific probe. Partial metaphase showing hybridization of the probe to the two normal 12s (*unlabeled arrows*) and the marker chromosome (*mar*). Note the greater signal intensity from the marker chromosome hybridization.

FIG. 4. *A. (middle, left)* Patient 4 karyotype: 46,XY,−7,+mar. *B. (middle, right)* C-bands: Partial metaphase with the C-band of the marker chromosome. *C. (lower left)* Fluorescent *in situ* hybridization with the chromosome 7–specific probe. Partial metaphase cell showing hybridization of the probe to the marker chromosome (*mar*) and to the normal chromosome 7 (*unlabeled arrow*). In this experiment, the background fluorescence intensity is partially increased.

centromeric DNA. Again, we cannot rule out the presence of other chromosomal DNA in the marker chromosome.

The patient was treated with vincristine, prednisone, and daunorubicin, and he entered complete remission. Four months later, in September 1990, he had morphologic and cytogenetic evidence of relapse.

DISCUSSION

Historically, determination of the origin of marker chromosomes in clinical cytogenetic studies has been a difficult but important task. A number of specialized techniques, such as C-banding, AgNOR staining, anti-

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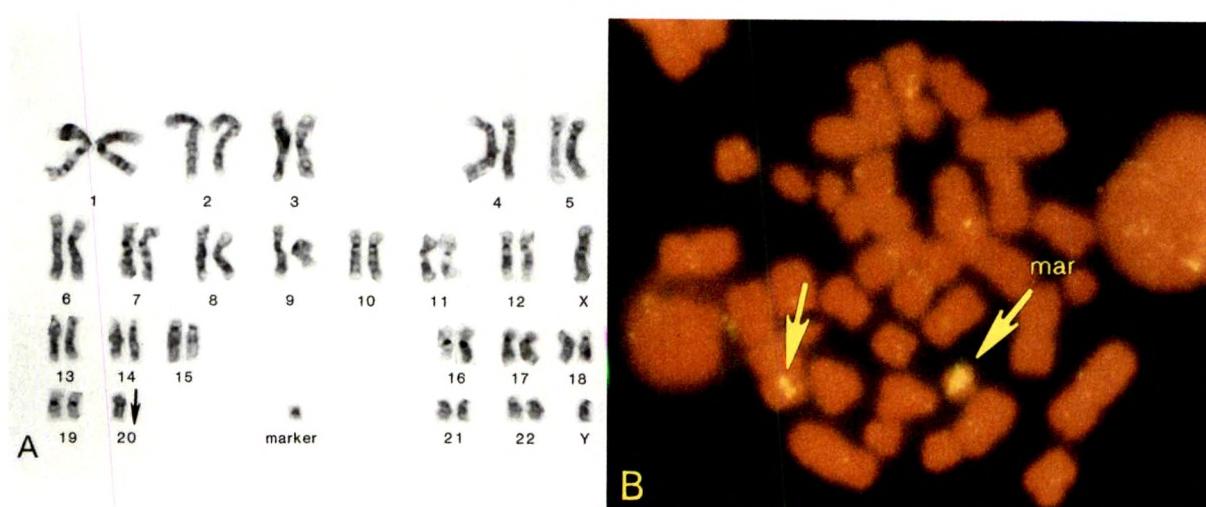

FIG. 5. *A.* Patient 5 karyotype: 46,XY,−20,+mar. *B.* Fluorescent *in situ* hybridization with the chromosome 20–specific probe. Partial metaphase showing hybridization of the probe to the normal chromosome 20 (*unlabeled arrow*) and to the marker chromosome (*mar*). In this experiment, the background fluorescence intensity is partially increased.

centromere staining, and distamycin/DAPI staining, have been developed to facilitate such identifications. However, many marker chromosomes cannot be identified with these techniques because there is too little chromosomal material to stain or the staining results are inconclusive. We are beginning to effectively use a battery of chromosome-specific DNA probes routinely for marker identification by fluorescent *in situ* hybridization.

Approximately 30 of the 3,800 PHA-stimulated lymphocyte and amniocyte studies performed in our laboratory during 1989 contained unidentifiable marker chromosomes. In the case of congenital abnormalities, studies on family members often help determine whether the marker chromosome is derived from a balanced chromosome abnormality. However, we have found that 90% of these cases are not resolved by family studies, either because of the *de novo* nature of the marker or familial reluctance to pursue the matter. Approximately 50% of the cases involve autosomal abnormalities, and the remainder have markers that involve the sex chromosomes. Most of the latter patients have a phenotype of Turner's syndrome or ambiguous genitalia. It is especially important in these cases to determine whether mosaicism of the sex chromosomes exists, because such patients have an increased susceptibility to gonadoblastomas.[17] In such cases, our laboratory has performed a Southern blot analysis of the patient's DNA to determine whether Y-specific DNA was present. However, this procedure necessitates a second phlebotomy, and the results take approximately two to three weeks to process. Fluorescent *in situ* hybridization, in addition to providing results that can be reported without delaying the cytogenetic study, also de-

termines whether the marker in question is derived from a specific chromosome.[18]

The fluorescent *in situ* hybridization results for patient 1 revealed that the marker chromosome is derived at least partially from an X chromosome. Because the Southern blot analysis verifies the absence of other Y-DNA in the genome, this patient has little risk for development of gonadoblastoma. However, the fluorescent *in situ* analysis of the marker and fragment chromosomes of patient 2 showed that both contained DNA of chromosome Y origin. Southern blot analysis indicated the presence of a significant amount of Y-DNA in the genome other than the centromeric DNA detected by the alpha-satellite probe. This patient is at risk for gonadoblastoma, and, despite her normal pubertal development, aided by the estrogen therapy, a prophylactic gonadectomy is being considered.

Cytogenetic analysis plays a critical role in the diagnosis and treatment of patients with neoplastic disorders. Hematologic studies generally produce a greater number of marker chromosomes than do congenital studies. Approximately 150 of the 3,300 bone marrow and unstimulated peripheral blood studies performed in our laboratory during 1989 contained marker chromosomes of indeterminate origin. In many cases, as in the three outlined here, the use of fluorescent *in situ* hybridization is crucial in determining the origin of the marker chromosome.

One year after diagnosis of a mediastinal germ cell tumor, patient 3 presented with ANLL. At the time of this diagnosis, two cytogenetic clones were found in the bone marrow. One clone was monosomy 7 with other trans-

Fluorescent In Situ Hybridization

locations, and a second clone contained a marker chromosome that resembled an i(12p). The patient underwent bone marrow transplantation, and subsequent chromosome cytogenetic studies were performed at the Mayo Clinic Cytogenetics Laboratory.

A single abnormal clone was found that had a marker chromosome that again resembled an i(12p). Fluorescent *in situ* hybridization studies with an alpha-satellite probe specific for the chromosome 12 centromere suggest that our initial impression was correct. This observation was significant because i(12p) has been associated with the metachronous appearance of mediastinal germ cell tumors and acute leukemias.[19] These tumors have been postulated to be derived from the same pluripotent stem cell.[20] These recent reports suggest that patient 3 indeed may have had this kind of tumor. An alternative explanation is that patient 3 inherited Pallister (Killian/Teschler-Nicola) syndrome, with its associated constitutional mosaicism for isochromosome 12p.[21-23] We cannot exclude this possibility, and it is important to mention that several patients with Pallister syndrome have had increased expression of the i(12p) within their bone marrow.[24] This might explain the coexistence of the isochromosome 12p and the lack of morphologic evidence of bone marrow relapse after the patient underwent bone marrow transplantation. In support of this alternative explanation, neoplastic clones with monosomy 7 have been associated with secondary MDS and secondary ANLL (reviewed in Yunis and Brunning[28]). When patient 3 presented with ANLL, his bone marrow contained a clone with monosomy 7 but lacked the i(12p) marker chromosome. Contrary to this alternative explanation, patients with Pallister syndrome never have been reported to have mediastinal germ cell tumors, to the best of our knowledge.[21,22,25,26] Unfortunately, patient 3 died before cytogenetic studies could be performed on skin fibroblasts to determine whether this alternative explanation is correct.

The clinical course of patient 4 progressed quite rapidly. Within two to four months, he progressed from having MDS resembling refractory anemia to having overt ANLL. This course is typical for patients with monosomy 7 or deletion 7q.[27,28] In patients with a ring chromosome 7 or a ring chromosome derived from a chromosome 7, a similar course might be predicted. The marker chromosome of patient 5 appeared to be an abnormal chromosome 20 in which portions of its short and long arms (20p− and 20q−) were deleted. Fluorescent *in situ* hybridization demonstrated that the marker was derived partially from a chromosome 20.

The usefulness of the fluorescent *in situ* hybridization method is limited, much like that of any other cytogenetic technique. One limitation is that the potential number of hybridization reactions can be quite large (24 to cover all chromosome centromeres). To select the appropriate probes, it helps to have some clues as to the origin of the marker chromosome (*e.g.*, a consistently missing chromosome within metaphases). The metaphases of patients 4 and 5 were missing a chromosome 7 and a chromosome 20, respectively. However, it is important to emphasize that these clues may not be helpful, especially in hematologic malignancies. Such marker chromosomes could just as well involve other chromosomes. The traditional specialized stains such as C-bands and distamycin/DAPI still may be necessary to minimize the number of hybridization reactions that must be performed. This limitation may be obviated by the development of automated methods, as well as the knowledge that some alpha-satellite DNA probes are known to cross-hybridize with several chromosomes. Thus, batches of chromosomes can be eliminated by the use of a single probe. If particular cross-hybridizing probes produce a positive result, then the appropriate chromosome-specific probes can be used under higher-stringency conditions.

A second limitation of this technique concerns the origin of DNA detected by the alpha-satellite DNA probe. These probes only identify the centromeric origin of a marker. Although in many cases it is likely that the remaining chromosome material is of the same origin as the centromere, the possibility exists that structural rearrangements involving other chromosomes have altered the noncentromeric portion of the marker, especially in complex bone marrow cases. In the analysis of patients 1 and 2, a Southern blot assay was necessary to determine the presence or absence of other Y-derived sequences in the patient's genome. For the analysis of patients 3-5, there is no assay that readily tests for the presence of significant portions of other chromosomes. It is important to note that acentric markers, by definition, would not be identifiable with the use of the alpha-satellite DNA probes.

Fluorescent *in situ* hybridization with chromosome-specific alpha-satellite DNA probes significantly expands the repertoire of the clinical cytogenetics laboratory for marker chromosome identification and thus provides a means for more accurate diagnosis.

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Single Case Report

Achromobacter xylosoxidans
An Unusual Neonatal Pathogen

YVONNE R. HEARN, M.D., AND RITA M. GANDER, PH.D.

Perinatal acquisition of a rare pediatric pathogen, *Achromobacter xylosoxidans,* with evidence for *in utero* transmission, is described. Cultures from the mother and neonate demonstrated *A. xylosoxidans.* An ascending bacterial infection in the mother with clinical chorioamnionitis is presented as the probable mode of transmission. Postmortem examination of the infant confirmed Achromobacter meningitis. In contrast to the current case with transmission from mother to neonate, previously published neonatal cases of Achromobacter infections indicate that nosocomial transmission of the organism is most common (79%). In addition, the literature review revealed a high mortality associated with meningitis (77%), frequent hydrocephalus, and subsequent neurologic sequelae (36%). To the authors' knowledge, this is the first documented case of maternal–fetal transfer of *A. xylosoxidans.* (Key words: *Achromobacter xylosoxidans;* Neonatal meningitis) Am J Clin Pathol 1991;96:211–214

Achromobacter xylosoxidans—an aquatic, aerobic gram-negative bacterium—primarily is considered to be an opportunistic pathogen and has been recognized as the etiologic agent in several pediatric nosocomial outbreaks.[1,2] In 1958, a cluster of nosocomial Achromobacter infections resulted in six deaths in a newborn nursery over a three-month period.[3] Seven other cases of neonatal meningitis have been reported in the literature.[4–6] The source of infection was not determined in most cases; however, nursery equipment was suggested as the possible mode of transmission in five of the seven cases. In contrast, community-acquired *A. xylosoxidans* infections are uncommon.

We describe a case of neonatal meningitis caused by *A. xylosoxidans* that was acquired perinatally. The source of the mother's community-acquired infection is unknown. This is the first documented case of transmission of *A. xylosoxidans* from mother to fetus.

REPORT OF A CASE

A 1,090-g female neonate of 27–28 weeks gestational age was born to a 28-year-old black woman (gravida 2, para 1). The mother was an un-employed, lifetime resident of Dallas county without a significant travel history. She presented at six to seven weeks gestational age with a urinary tract infection. *Acinetobacter calcoaceticus* var. *anitratus* was recovered from her urine. She was not treated, and a follow-up urine culture was negative. At approximately 25 weeks gestation, beta-hemolytic gram-positive cocci were recovered in low numbers from a urine sample. Her physician prescribed 500 mg ampicillin per day for a total of seven days, but she did not comply with the oral ampicillin regimen. Approximately three weeks later, the woman presented in early labor with presumed chorioamnionitis. She was treated with intravenous ampicillin. After 12 hours of labor, her membranes were ruptured artificially, revealing purulent amniotic fluid. Unfortunately, the amniotic fluid was not cultured. Gentamicin and clindamycin were added to the antibiotic regimen.

Shortly thereafter, the patient spontaneously delivered a hydropic female infant with Apgar scores of 5 at 1 minute and 7 at 5 minutes. The child required intubation for decreased heart rate and absence of spontaneous respiration. Initial assessment included hydrops of unknown origin, prematurity, respiratory distress, and possible sepsis. The neonate was treated with ampicillin, gentamicin, and ceftazidime. On the second day of life, pulmonary hemorrhage, bilateral grade 3 intraventricular hemorrhages, and gastrointestinal hemorrhage developed in the infant. Subsequently, *A. xylosoxidans* was recovered from two sets of blood cultures from the neonate, and the antibiotic regimen was changed to ticarcillin/clavulanate and gentamicin. The infant's course was complicated, and her condition continued to deteriorate. Despite maximal support, the infant died on the eighth day.

Before delivery, a blood sample from the mother also showed *A. xylosoxidans.* Clinically, the mother's condition improved dramatically in the immediate postpartum period. After three days of hospitalization, she was discharged while being treated with amoxicillin/clavulanate. She was lost to follow-up.

PATHOLOGIC FINDINGS

At autopsy, examination of the neonate revealed extensive anasarca, early bronchopulmonary dysplasia,

cloudy basilar leptomeninges with microscopic purulent meningitis, bilateral subependymal hemorrhage, cerebellar hemorrhage, significant thymic stress involution, and widespread extramedullary hematopoiesis. The placenta was not available for examination.

As mentioned previously, cultures from both the mother and neonate demonstrated *A. xylosoxidans*—including a culture of a blood specimen drawn from the mother 42 hours before delivery; and cultures of antemortem blood specimens drawn from the neonate at 6 hours of age and 40 hours of age, and a postmortem meningeal culture. The bacterial isolates were identified as *A. xylosoxidans*, with the use of the Vitek Systems gram-negative identification cards (Vitek Systems, Hazelwood, MO). The identifications were further confirmed by electron microscopic examination; peritrichous flagella were observed on the bacterial cells. The three isolates from the neonate and the single isolate from the mother demonstrated identical biochemical reactivities and antimicrobial susceptibility patterns. The bacterial isolates were susceptible to amoxicillin/clavulanate, ticarcillin/clavulanate, trimethoprim–sulfamethoxazole, and imipenem. There was *in vitro* resistance to ampicillin, ampicillin/sulbactam, piperacillin, mezlocillin, tetracycline, cefuroxime, cefoxitin, ceftriaxone, ceftazidime, amikacin, gentamicin, tobramycin, chloramphenicol, aztreonam, and ciprofloxacin. Other microbiologic studies, including viral cultures and tests to detect bacterial antigens, did not reveal additional infectious causes in the mother or neonate.

DISCUSSION

Achromobacter xylosoxidans is an uncommon pediatric pathogen. Most *A. xylosoxidans* infections occur in high-risk patients, such as compromised hosts and neonates.[2] Neonatal infections usually include bacteremia and meningitis, followed by death.[2] The clinical and autopsy evidence for the current case documents a neonatal *A. xylosoxidans* infection.

The source of the mother's community-acquired Achromobacter infection is unknown. Epidemiologic data suggest that water is the natural reservoir of *A. xylosoxidans*. In the community, this organism has been recovered from well water[7] and swimming pools.[8] Achromobacter is not found on fomites and does not appear to exist in nonaquatic habitats.[2]

The mother was bacteremic with *A. xylosoxidans* 42 hours before delivery. In addition, the isolation of a similar organism from the infant's blood at 6 hours of life and subsequently at 40 hours documented *A. xylosoxidans* bacteremia in the neonate. Therefore, it appears that this bacterium was acquired *in utero*. An ascending infection involving the birth canal, followed by chorioamnionitis and subsequent involvement of the neonate, appears to be the most plausible explanation.

We speculate that the etiologic agent, *A. xylosoxidans*, may have originated in the mother's vagina or urinary tract.[2,9] An ascending infection, as proposed in this case report, may be predisposed by a symptomatic or asymptomatic maternal urinary tract infection.[9] The mother had a history of recurrent urinary tract infections throughout her pregnancy. However, *A. xylosoxidans* was not recovered from her urine when she was admitted to the hospital. A possible explanation is that the organism was present, but in numbers undetectable by standard culture methods. Women of childbearing age can have urinary tract infections and significant bacteriuria of less than 10^3 organisms per milliliter.[10]

Chorioamnionitis often is associated with membranes that have been ruptured for more than 24 hours. In contrast, in this case the mother's membranes were ruptured artificially approximately three hours before delivery. However, acute chorioamnionitis may occur infrequently with intact fetal membranes or membranes that have been ruptured for less than 24 hours.[11]

Alternatively, blood-borne microorganisms may have infected the placenta, with secondary development of chorioamnionitis. Transplacental or hematogenous infections that result in villositis usually include only syphilis, listeriosis, tuberculosis, viral infections, toxoplasmosis, and malarial infections. There are only rare cases in which other microorganisms reach the placenta by the hematogenous route. Pyogenic organisms produce septic infarcts and extensive villositis when the placenta is involved.[11] Placental examination would clarify this issue; however, the placenta from our case was not available.

Another possible route of infection was through a colonized birth canal.[3] *A. xylosoxidans* may be part of the normal colonic flora of some people and has been recovered infrequently from the vaginas of some women.[2] Theoretically, this neonate could have become colonized with *A. xylosoxidans* through the vaginal and colonic flora. However, considering the remoteness of perinatal or transplacental transmission of this bacterium, the most probable route of infection in this case was an ascending infection with subsequent chorioamnionitis.

Including the current case, a total of 14 cases of neonatal Achromobacter infection have been reviewed.[4–6,12] The pertinent findings in these cases are summarized in Table 1. The early onset of symptoms, positive antemortem cerebrospinal fluid culture, prematurity, high frequency of fatal outcome, and development of hydrocephalus with neurologic sequelae were the predominant features.[4–6,12] The clinical features associated with Achromobacter infection in neonates are generally nonspecific. However,

TABLE 1. REPORTED CASES OF NEONATAL INFECTIONS WITH *ACHROMOBACTER XYLOSOXIDANS*

Case No.	Estimated Gestational Age (weeks)	Onset of Symptoms* (days)	Recovery of Achromobacter from Clinical Specimens†	Outcome of Infection	Author Year
1	33	1	—	Patient died	Foley and associates. 1961[12]
2	35	1	Postmortem blood	Patient died	Foley and associates. 1961[12]
3	32	1	—	Patient died	Foley and associates. 1961[12]
4	39	1	Antemortem and postmortem blood	Patient died	Foley and associates. 1961[12]
5	36	1	Nose, throat, blood	Patient died	Foley and associates. 1961[12]
6	32	1	Nose, throat, blood	Patient died	Foley and associates. 1961[12]
7	40	2	CSF, blood	Patient survived	Sindhu, 1971[6]
8	—	<1	CSF	Unknown‡	Sindhu, 1971[6]
9	Premature	16	CSF	Patient died	Sindhu, 1971[6]
10	40	6	CSF	Unknown§	Lee and Tan, 1972[4]
11	—	<1	CSF	Patient died	Lee and Tan, 1972[4]
12	37	6	CSF, blood	Patient died	Lee and Tan, 1972[4]
13	33	<1	CSF	Patient died	Namnyak and associates, 1985[5]
14	30	<1	Antemortem and postmortem blood, postmortem meninges	Patient died	This study

* Length of time following birth before onset of symptoms.
† Antemortem clinical specimens, unless otherwise noted.
‡ Patient alive and well at 20 weeks of age; subsequently lost to follow-up.

§ Patient discharged at 6 weeks of age with hydrocephalus and lead pipe rigidity; subsequent ly lost to follow-up.

the mortality rate of 77% and the frequent development of neurologic sequelae are particularly striking. Only one long-term survivor was documented in the literature. Two additional infants were lost to follow-up after antimicrobial therapy was completed.

In the published cases, the sources of infection were not determined in most instances, except in the study of Foley and associates, which describes six deaths in a newborn nursery.[4-6,12] Although the mode of transmission was not proven, the available data suggested that the water used for washing the infants' eyes probably contained the organism.[12] Most of the previously reported cases were proposed to be nosocomial. For three of the neonatal Achromobacter infections, it was suggested that contaminated incubators were the source of the microorganisms. However, this was not documented.[6] Two of three Achromobacter cases reported by Lee and Tan were linked to unspecified hospital nursery contaminants.[4] In the study of Lee and Tan, a maternal source was suggested for one of the neonatal infections, based on the early onset of symptoms after birth.[4] In the case report of Namnyak and co-workers, describing neonatal Achromobacter meningitis, a community-acquired infection could not be excluded because the infant was delivered at home.[5] In summary, nosocomial acquisition is presumed in 11 of 14 reported cases. A maternal source of infection is proposed in only one case, other than the current one.[4-6,12]

Some confounding variables affect proper analysis of the previously published reports, including nomenclature, microbiologic technique, and absence of postmortem examinations. The designation "Achromobacter" is considered controversial. *Bergey's Manual of Systematic Bac-*

teriology does not accept "Achromobacter" as a genus.[13] This microorganism is listed in Bergey's as *Alcaligenes denitrificans* subspecies *xylosoxidans*.[14] However, the name "*Achromobacter xylosoxidans*" is recognized by experts at the Centers for Disease Control and by most clinical microbiologists.[13,15,16] Thus, the controversial nomenclature makes interpretation of some of the older literature difficult.

In addition, techniques for the identification of nonfermentative gram-negative bacilli vary from institution to institution. Many laboratories rely on commercial systems that may demonstrate diminished accuracy when used to identify Achromobacter species. Achromobacter isolates show restricted biochemical reactivity, oxidizing only a small number of carbohydrates, including xylose[14] Because of its limited biochemical characteristics, Achromobacter species may be confused with Pseudomonas species with the use of these commercial systems. However, the genus can be distinguished easily from Pseudomonas species when biochemical data are combined with morphologic observations. The former demonstrate peritrichous flagella, whereas the latter show a polar distribution of flagella.[14]

Treatment regimens in the reported cases varied considerably, making comparisons difficult.[4-6,12] In the neonate we described, ampicillin, gentamicin, and ceftazidime were administered empirically. The infant's bacterial isolates were resistant to these three agents. Later, the antibiotic regimen was changed to ticarcillin/clavulanate and gentamicin. Results from *in vitro* susceptibility tests demonstrated that the Achromobacter isolates from the neonate were susceptible to ticarcillin/clavulanate. The ini-

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tial use of potentially ineffective antibiotics may have contributed to the neonate's death.

In contrast, the mother's condition improved clinically while she was receiving ampicillin and gentamicin. However, the bacterial isolate recovered from her also demonstrated *in vitro* resistance to these two antimicrobials. *In vitro* results do not always correlate with clinical outcome. The combination of ampicillin and gentamicin may have acted synergistically *in vivo*. This possible enhancement of antimicrobial activity would not be reflected in the *in vitro* susceptibility results. A more remote explanation for the mother's apparent clinical response to potentially ineffective agents is that the mother was transiently bacteremic. The organism was only isolated from a single blood culture from the mother.

An antipseudomonal penicillin or a third-generation cephalosporin combined with trimethoprim–sulfamethoxazole have been recommended as the drugs of choice for *A. xylosoxidans* infections, pending susceptibility testing.[2,17] Isolates of *A. xylosoxidans* generally are resistant to ampicillin, cephalothin, and the commonly used aminoglycosides.[2,12]

As mentioned above, *A. xylosoxidans* has been confused occasionally with *Pseudomonas aeruginosa*.[1,2,16,18] An Achromobacter infection should be considered in the perinatal period when a nonfermentative gram-negative bacillus resistant to aminoglycosides is recovered from a neonate. Because some common antipseudomonal agents such as aminoglycosides are not effective against *A. xylosoxidans,* it is imperative to make the distinction between Pseudomonas and Achromobacter.[1,2,16,18]

In summary, the infant described contracted *A. xylosoxidans* while *in utero,* probably through chorioamnionitis, and died subsequently. To our knowledge, this case illustrates the first example in the literature of proven congenital acquisition of *A. xylosoxidans.* Furthermore, this case reminds physicians to include this bacterium in a differential diagnosis of neonatal infections, especially if a Pseudomonas-like organism is suspected.

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Congenital Atransferrinemia

A Case Report and Review of the Literature

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A four-year-old Polynesian girl with a two-year history of severe microcytic, hypochromic anemia (which was refractory to iron therapy) had a decreased beta-globulin fraction on serum protein electrophoresis, resulting from the absence of the transferrin (TRF) band. Subsequent assays for TRF showed a level below the detectable range. Liver biopsy revealed significant deposition of hemosiderin within hepatocytes and Kupffer cells, in addition to early fibrosis. Two bone marrow aspirates were hypercellular, with decreased myeloid–erythroid ratios. This case represents the eighth reported example of congenital atransferrinemia, a rare, apparently autosomal recessive disease. (Key words: Congenital atransferrinemia; Anemia; Hemosiderosis; Therapy) Am J Clin Pathol 1991;96:215–218

Congenital atransferrinemia (CAT) is an extremely rare, recessively inherited disorder. To date, only seven cases have been reported in the world literature. Transferrin (TRF) is a glycoprotein with a single polypeptide chain and a molecular weight of approximately 90,000 daltons, that mediates the transfer of hemoglobin iron and absorbed iron to cells and storage sites.[1] Patients who have CAT or acquired atransferrinemia (AT) have moderate to severe microcytic, hypochromic anemia, with normal serum iron levels, high serum ferritin values, and a significantly low total iron-binding capacity (TIBC). Intestinal iron absorption continues, and iron overload can be expected to develop in all patients. We report an additional case of CAT in a four-year-old Polynesian girl from American Samoa, along with the literature on this topic.

REPORT OF A CASE

In April 1989, a four-year-old Samoan girl was referred to the Pediatric Hematology/Oncology Service at Tripler Army Medical Center for evaluation of recurrent anemia associated with significant hepatosplenomegaly. The child's medical history included chronic hypochromic, microcytic anemia, which had required transfusion therapy on at least three occasions. She had been given iron several times without benefit. About three weeks before her transfer to Honolulu, the child was seen in the Pediatric Clinic of another institution in Pago Pago, American Samoa. At that time, she was febrile, with mild respiratory symptoms. She also had significant hepatosplenomegaly. A complete blood count was performed, and her hemoglobin level was 40 g/L (4.0 g/dL). She received transfusions of whole blood, and, subsequently, she was transferred to our hospital. When she arrived, additional history was obtained from her mother, who denied a family history of anemia. Additionally, the patient's mother stated that the child had no known history of blood loss and that multiple tests to detect blood in the stool had been negative.

LABORATORY FINDINGS

The hemoglobin level was 50 g/L (5.0 g/dL), with a mean corpuscular volume of 66.7 fL (66.7 μm^3), a mean corpuscular hemoglobin concentration of 313 g/L (31.3 g/dL), and an absolute reticulocyte count of 104×10^9/L (104,000 mm^{-3}). The white blood cell count was 6.9×10^9/L (6,900 mm^{-3}), with a normal differential analysis, and the platelet count was 555×10^9/L (555,000 mm^{-3}). Abnormal forms included teardrops, ovalocytes, and target cells. Serum electrolytes, liver enzymes, and renal function tests were normal. Iron studies showed a total iron concentration of 3.40 $\mu mol/L$ (19 $\mu g/dL$) (normal, 65–175 $\mu g/dL$), TIBC of 12.36 $\mu mol/L$ (69 $\mu g/dL$) (normal, 250–410), and ferritin level of 783 $\mu g/L$ (783 ng/mL). Serologic results for hepatitis B virus, hepatitis A virus, Toxoplasma, and cytomegalovirus were negative. Serum protein electrophoresis showed a significantly decreased beta-globulin fraction, resulting from the absence

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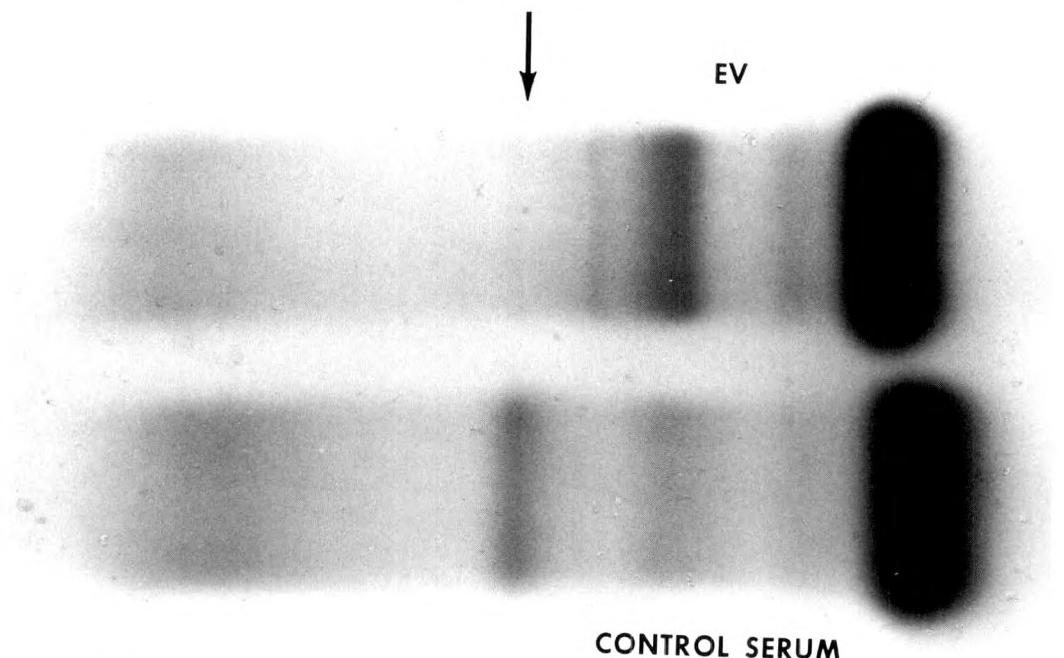
FIG. 1. Serum protein electrophoresis strip. *Arrow* marks normal location of the transferrin band, which is easily visualized in the control.
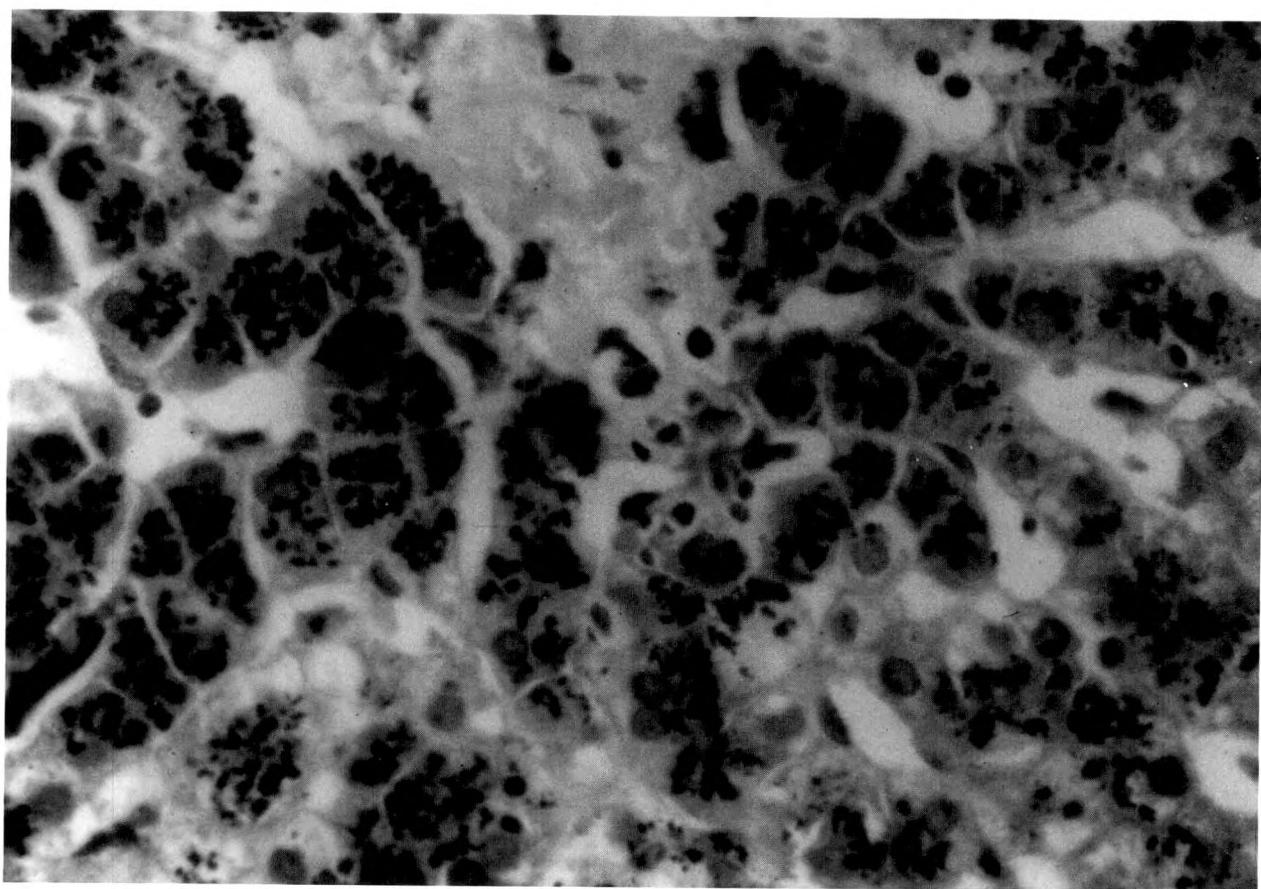
FIG. 2. Liver biopsy with marked hemosiderosis. Pearl's iron stain (×600).

of the TRF band (Fig. 1). TRF was not detected by nephelometry. The mother's TRF level was 1.59 g/L (159 mg/dL) (normal, 220–400 mg/dL).

A bone marrow aspirate was hypercellular, with a decreased myeloid–erythroid ratio. Iron stores were decreased. To assess the patient's hepatomegaly, a percutaneous liver biopsy was performed. There was a striking amount of Prussian blue–positive golden brown pigment (iron) in the hepatocytes and Kupffer cells (Fig. 2). Early bridging fibrosis was focally present.

DISCUSSION

Transferrin (siderophilin) is a glycoprotein with a molecular weight of approximately 90,000 daltons and consists of a single polypeptide chain. It is synthesized primarily in the liver, but the reticuloendothelial system (RES) and some endocrine glands also have the capacity to produce TRF. In the plasma, TRF is bound not only to iron, but also to copper, zinc, calcium, and cobalt. A single TRF molecule binds two molecules of iron in association with an anion, which usually is bicarbonate. Iron is derived from catabolized hemoglobin and intestinal absorption. TRF then releases the iron for integration into ferritin and hemosiderin in the RES and into cells that synthesize iron-containing compounds (hemoglobin, myoglobin, cytochromes).[1]

Transferrin can be assayed by nephelometry or radioimmunodiffusion and has a normal range of 2.0–4.0 g/L (200–400 mg/dL). Another crude measurement of TRF is the TIBC, but it will overestimate the actual TRF because other plasma proteins such as albumin also bind iron.[2]

Because TRF is a negative acute-phase reactant, low levels occur in many inflammatory and neoplastic conditions. Other conditions, such as liver disease (decreased synthesis) and nephropathies or enteropathies (increased loss), also can lead to decreased serum levels. In some cases, the levels are as low as those in CAT.[3-5] However, the complete absence or trace levels of TRF are exceedingly rare, with only seven cases having been reported.[4,6-10]

In these examples (Table 1), the children had severe microcytic, hypochromic anemia, with a low iron level and a low TIBC but elevated ferritin levels. Serum TRF levels ranged from undetectable to 0.39 g/L (39 mg/dL).[9] No other associated congenital anomalies or syndromes have been reported with this disorder. In addition to the severe anemia, there are major findings related to iron overload, which often is exacerbated by treatment with iron or blood transfusions. Symptoms are associated with increased iron deposition in the liver and RES. The patients of Heilmeyer and associates and Dorantes-Mesa and associates and our patient all had severe hepatic hemosiderosis that was documented by liver biopsy or autopsy.[4,7] The second of these patients also had splenic involvement. However, treatment with iron or blood transfusions probably increased the amount of iron deposition in these three cases.

The exact cause of CAT is not entirely clear, but, because parents of all of the affected children have low levels of TRF, transmittance of an autosomal recessive trait is suggested strongly. The putative carriers have no symptoms and are not anemic.[7,8,10] Thorough evaluation of additional cases of CAT or DNA analysis might help clarify this issue.

Purified human TRF, as used by Kawakami and associates, has been effective in the treatment of CAT.[11] One patient had a dramatic increase in hemoglobin.[11] Unfortunately, preparation of TRF is difficult and expensive. Other treatment modalities include transfusion of fresh-frozen plasma and the use of iron chelators. Our patient was treated with the latter two modalities, with a significant increase in hemoglobin concentration and increased urinary excretion of iron. Of course, erythrocyte transfusions are contraindicated, because these increase iron storage.

Data are limited on the prognosis of patients with CAT. As stated previously, three of six patients had extensive

TABLE 1. HEMATOLOGIC AND IRON METABOLISM DATA ON CAT CASES

Author	Year	Age/Sex	Hemoglobin g/L (g/dL)	Iron μmol/L (μg/dL)	TIBC (μmol/L (μg/dL)	Transferrin g/L (mg/dL)	
						Patient	Parents M/F
Heilmeyer[4]	1961	7/F	96 (9.6)	1.61 (9)	5.91 (33)	0.044 (4.4)	
Cap[6]	1968	11 mos/F	48 (4.8)	5.37 (30)	5.37 (30)	None	
Sakata[9]	1969	10/F	32 (3.2)	2.87 (16)	14.51 (81)	0.390 (39)	
Goya[8]	1972	8/M	64 (6.4)	2.15 (12)	8.24 (46)	Trace	0.800 (80)/1.060 (106)
Walbaum[10]	1971	7/F	91 (9.1)	2.51 (14)	3.58 (20)	None	Normal/Normal
Dorantes-Mesa[7]	1986	9/F*	79 (7.9)	3.40 (19)		None	1.100 (110)/1.150 (115)
Dorantes-Mesa[7]	1986	3/F*	79 (7.9)			0.062 (6.2)	
Hamill (this study)	1990	4/F	50 (5.0)	3.40 (19)	12.56 (69)	None	1.59 (159)/—

* Siblings.

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hemosiderosis of the liver, and one died. At the time of the case report, one patient who received purified TRF was alive after 17 years.[11] Untreated patients probably will die early from hemochromatosis or congestive heart failure.

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Original Article

Poorly Differentiated Adenocarcinoma with Lymphoid Stroma (Lymphoepithelioma-like Carcinomas) of the Stomach

Report of Three Cases with Epstein–Barr Virus Genome Demonstrated by the Polymerase Chain Reaction

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AND TIMOTHY J. O'LEARY, M.D., PH.D.[3]

Lymphoepithelioma of the nasopharynx is an undifferentiated carcinoma with prominent lymphoid infiltration. Histologically similar tumors have been documented in the skin, lung, thymus, salivary gland, tonsil, and uterine cervix. The authors report three cases of gastric carcinoma that were histologically and immunohistochemically similar to lymphoepithelioma. The patients were elderly white persons (61, 76, and 77 years of age); two of them had previous partial gastric resections for benign ulcer disease. The tumors were located on the lesser gastric curvature (two cases) and at a previous anastomotic site (one case) and measured 3, 4, and 7 cm in largest dimension. Grossly, each neoplasm was a raised plaque-like lesion with a central ulcer. Microscopically, all three tumors were similar, with poorly differentiated polygonal tumor cells scattered throughout a dense lymphocytic background. In a few areas, tumor cells formed ill-defined cords. In two cases, the neoplasms invaded the gastric muscle layer but had a well-delineated (pushing) margin. The tumor cells were immunohistochemically positive for AE3-defined keratin, confirming their epithelial nature. The lymphocytes were a mixture of UCHL-1-positive T cells and L-26-positive B cells. Portions of the Epstein–Barr virus genome were present in all cases, as detected by the polymerase chain reaction. The morphologic features of these cases are similar to those of lymphoepithelioma in other anatomic sites, and these tumors represent a unique subtype of gastric carcinoma. (Key words: Lymphoepithelioma-like carcinoma; Epstein–Barr virus; Gastric carcinoma; Polymerase chain reaction) Am J Clin Pathol 1991;96:219–227

Lymphoepitheliomas (LEs) of the nasopharynx have specific histologic, immunohistochemical, and ultrastructural features.[1,2] By light microscopic examination, LE appears as poorly differentiated malignant cells that are arranged in cords or syncytial masses, or admixed singly with a prominent lymphoid stroma. The latter may be so pronounced that the lesion can be mistaken for a lymphoma, justifying its designation as LE.[3] Elegant histopathologic analyses by many investigators, including Willis,[4] have shown that the malignant elements in LE are poorly differentiated squamous cells. Using electron microscopic[2,5] and immunohistochemical techniques,[1,6] other investigators recently confirmed their squamous epithelial nature by demonstrating abundant cytoplasmic tonofilaments and strong immunoreactivity for cytokeratin. These findings led to the conclusion that LEs represent poorly differentiated carcinomas in the spectrum of squamous carcinomas of the nasopharynx. However, some studies have claimed that the neoplastic cells in LE closely resemble those of tonsillar crypts,[7] suggesting the possibility that LEs arise from crypt cells. This contention is in contradistinction to that attending common pharyngeal carcinomas, which are thought to arise from the surface squamous epithelium.

Compared to squamous cell carcinomas, LEs occur in young patients and are more common in Chinese patients, who often are from Hong Kong.[4,7] The latter finding suggests the possibility that certain infectious agents might be involved in their pathogenesis. In 1970, zur Hausen and co-workers[18] reported that Epstein–Barr virus (EBV) was associated with LE of the nasopharynx, and this observation has been confirmed by other authors.[8–10] These epidemiologic data have justified the separation of LEs from common nasopharyngeal squamous cell carcinomas.

Lymphoepithelioma-like carcinomas have been reported in the skin,[11] lung,[12] thymus,[13] salivary gland,[14] uterine cervix,[15] stomach,[16] and larynx,[17] in addition to the nasopharynx. In some of these cases, parts of the EBV genome have been demonstrated in neoplastic cells, suggesting that the tumors resemble nasopharyngeal LEs, not only morphologically but also pathogenetically.

We report three cases of LE-like carcinoma of the stomach that contained demonstrable EBV viral genome, as shown by the polymerase chain reaction (PCR). Two different highly conserved sequences of EBV-DNA were seen in these lesions.

MATERIALS AND METHODS

Two cases of gastric carcinomas (cases 1 and 2) with histopathologic features of LE were accessioned during a nine-month period at the Oklahoma Medical Center, and another case (case 3) was received in consultation. For routine histologic examination, tissue was fixed in 10% buffered formalin. Paraffin sections were stained with hematoxylin and eosin, and immunostains were performed by incubating sections with the primary antibodies L-26, UCHL-1, anti-carcinoembryonic antigen (CEA; DAKOPATTS, Carpinteria, CA), and AE1 (Signet, Dedham, MA) and AE3 (Signet). Specific antibody-binding sites were demonstrated by applying a biotinylated, affinity-purified antimouse, rat, guinea pig, and rabbit IgG "cocktail" and streptavidin–peroxidase complex, with 3-amino 9-ethylcarbazole as the chromogen.

In Situ *Hybridization (ISH)*

Five-micron–thick paraffin sections were mounted on adhesive precoated slides and baked for one hour at 60°C. After dewaxing and rehydration with deionized water, sections were digested with proteinase for 10 minutes. After three 1-minute washings with wash buffer, the sections were soaked in Quench Reagent for 10 minutes. The sections were washed and dehydrated through a series of graded alcohol and dried completely. Next the sections were heated on a 92°C heating block with EBV Pathogene (Enzo, New York, NY) for 5 minutes and incubated on

a 37°C heating block for 30 minutes. Then 0.5 mL of Post Hybridization Reagent (Enzo) was applied to the section for 10 minutes. Hybridized sites were demonstrated by the ABC peroxidase technique. Human placental genomic probe was used as positive control and no DNA probe was used for negative control.

Polymerase Chain Reaction

Polymerase chain reaction amplification of the EBV genome was performed by two different methods:

PCR Method 1. The procedure for PCR amplification using paraffin tissue sections was modified from that of Shibata and associates.[17] Two 6-μm sections of tissue were cut from each block and placed in an Eppendorf tube. A fresh microtome blade was used for every specimen to avoid carryover of DNA from one specimen to the next. The paraffin was dissolved by vortexing the sections in 800 μL xylene. After addition of 400 μL ethanol, each tube was vortexed again and centrifuged at 14,000 rpm for 5 minutes. The supernatant was decanted, and the sample was resuspended by vortexing in 800 μL ethanol. After another 5-minute centrifugation, the supernatant was decanted again, and any remaining ethanol was removed with a microcapillary pipet. The sample was resuspended in 100 μL nonionic detergent buffer (50 mmol/L KCl, 10 mmol/L TRIS–HCl [pH 8.3], 2.5 mmol/L MgCl$_2$, 0.45% Nonidet P-40®, and 0.45% Tween 20®), to which 2.4 μL 2.5 mg/mL Proteinase K was added just before use. The sample then was incubated at 55 °C for one hour and 95 °C for 10 minutes to inactivate the enzyme. The tube subsequently was centrifuged for 5 minutes. A 6-μL aliquot of the supernatant was removed and diluted with buffer to a total of 15 μL; 0.5 μL and 2.5 μL of this suspension were added to the PCR reaction mixture, representing 1/250 and 1/50, respectively, of the DNA extracted from each 6-μm tissue slice. (Generally, approximately 80% of cases give a positive signal for the control HER2 gene at these dilutions.) Cases that were negative after the initial PCR were retested using the equivalent of one fifth to one tenth of the DNA in a 6-μm section.

The PCR reaction mixture contained 5 μL 10× Taq polymerase buffer (500 mmol/L KCl, 100 mmol/L TRIS–HCl, 15 mmol/L MgCl$_2$, and 0.1% gelatin), 200 μmol/L dNTPs, 100 nmol/L EBV primer, or 150 nmol/L HER2 primer, and one unit Taq polymerase in a final reaction volume of 50 μL. Fifty microliters mineral oil was layered over the reaction mixture. After an initial 5-minute incubation at 95 °C, the samples were subjected to 40 PCR cycles lasting 1-minute each at 95 °C, a 1-minute cycle at 55 °C, and two cycles at 72 °C for 7 minutes. Then

they were cooled to 4 °C. The MgClA segment of the EBV-DNA IR3 region was selected for identification. The primers and probe for this sequence were designed by Synthetic Genetics Company and have been described in its catalog (Table 1). This primer pair gives rise to a 240-base pair amplified product. The primers and probes were selected, based on a published partial sequence for the gene (Table 1), and give rise to a 241-base pair amplified product. Paraffin-embedded cells from an EBV-containing Burkitt's lymphoma (Raji) cell line were used as a positive control for EBV-DNA. Negative control material consisted of DNA extracted from a histiocytic lymphoma cell line (SUDHL5), which was obtained from Dr. Alan Epstein. In addition, negative controls for reagent contamination, consisting of all PCR reagents except template DNA, were used each time a PCR assay was performed.

Eighteen microliters of the PCR product and 2 μL loading buffer were electrophoresed in 2% NuSieve® agarose gel (FMC Bioproducts) and 0.5% agarose gel (Bethesda Research Laboratories, Bethesda, MD) for 90 minutes to two hours at 100 V. The gels were blotted overnight on an Oncor® nylon membrane, prehybridized for 30 minutes at room temperature in Oncor Membrane Blocking Solution®, and hybridized for four hours at 45 °C in 10 mL Oncor Hybrisol III® with 100-200 ng end-labeled probe. (Oligonucleotide probes were prepared by 5' end labeling with the use of ^{32}P-adenosine triphosphate and T4 DNA kinase.) The membrane then was washed twice (each wash was 5 minutes) at room temperature and once for 10 minutes at 55 °C, with 0.16× SSC and 0.1% SDS. Clear bands were generally visible after 4-16 hours of exposure on Kodak XAR5® film (Eastman Kodak, Rochester, NY).

PCR Method 2. Specific amplification of a segment of the long internal direct repeat region of EBV-DNA was achieved with the PCR as described previously.[9] Briefly, crude DNA templates were extracted from a single 8-μm section of formalin-fixed, paraffin-embedded tissue by successive cycles of deparaffinization with xylene and rehydration with ethanol, as described by Shibata and associates.[17] The dried pellet was boiled in buffer containing 10 mmol/L TRIS-HCl (pH 8.0) and 1 mmol/L ethylene

TABLE 1. PRIMERS AND PROBE USED FOR PCR

Epstein-Barr Virus Primers and Probe

Sense primer:
  5' GACGAGGGGCCAGGTACAGG 3'
Antisense primer:
  5' GCAGCCAATGCTTCTTGGACGTTTTTGG 3'
Probe:
  5' CGTCCTCGTCCTCTTCCCCGTCCACGTCCATGGTTATC-
  ACC 3'

diaminetetraacetic acid and centrifuged, and the supernatant was analyzed for the presence of EBV templates by the PCR. DNA from Raji cells and normal placenta served as positive and negative control templates, respectively. The DNA samples were incubated using the PCR, with oligonucleotide primers designed to amplify a 110-base pair segment of the long internal repeat region of EBV, as well as deoxynucleotide substrates, buffer, and Taq polymerase. Thirty cycles of amplification were obtained with a Perkin-Elmer thermal cycler (Perkin-Elmer-Cetus, Norwalk, CT). Amplification products were separated electrophoretically and transferred to nylon membranes. The resulting blots were hybridized with an oligonucleotide probe that was specific for the amplified segment. Samples were considered to be positive if the probe annealed with a 110-base pair species. Analyses were not considered valid unless all negative control reactions (containing placental DNA or buffer in the place of template) yielded negative results. In addition to negative and positive controls, three cases of gastric carcinomas of ordinary histologic features randomly selected from cases diagnosed during the same period also were studied and served as controls.

RESULTS

Summary of Clinical and Microscopic Features

Additional clinicopathologic data from our cases are shown in Table 2. In summary, each patient was an elderly white person with primary carcinoma of the stomach. None had nasopharyngeal carcinoma or evidence of metastatic disease. Two had partial gastrectomies in the distant past (35 years, 15 years) for benign ulcer disease. At follow-up, two patients were well 17 and 24 months after surgery, respectively. The third patient died of heart failure and sepsis one month after partial gastrectomy.

The tumors ranged in size from 3 to 7 cm in length and from 0.5 to 1 cm in thickness. The gross appearances of the tumors were remarkably similar. Each tumor was a raised plaque-like lesion with relatively well-demarcated, pushing borders. The neoplasms were limited almost entirely to the submucosa, with only superficial erosion of muscularis (Fig. 1). The cut surfaces were grayish and granular, with superficial ulceration.

The microscopic appearances of all tumors also were similar. Poorly differentiated malignant cells were dispersed throughout a dense lymphocytic background (Figs. 2 to 4). Occasional syncytial aggregates or ill-defined cords of tumor cells were seen; however, most of them were singly dispersed or clustered in small groups. The tumors were well circumscribed, with pushing margins (Fig. 4). In one case the neoplasm was limited to the submucosa.

Original Article

TABLE 2. CLINICOPATHOLOGIC DATA

Age/Race/Sex	Clinical History Surgical Procedure	Gross Findings	Histologic Findings	Follow-up
Case 1 77/W/F	A. Dysphagia B. Sister died of perforated ulcer, no known history of ulcer disease	2 lesions A. 4.0 cm. Posterior wall lesser curvature central ulcer B. Raised nodular esophagogastric junction	Poorly differentiated extension to the inner part of muscularis. 22 lymph node negative	Died of congestive heart failure and sepsis 1 month after surgery. No autopsy
Case 2 76/W/F	A. 50% gastrectomy for benign ulcer disease B. Anemia	3.0 × 2.5 × 0.5 cm, firm, central ulcer. Lesser curvature	Poorly differentiated with a focus of gland formation on the surface limited to the submucosa. 1 lymph node negative	1 month postoperation. Deep venous thrombosis. Alive and NTR
Case 3 61/W/M	A. Partial gastrectomy—benign ulcer disease (1974) B. Anemia, angina, nonspecific gastrointestinal disturbances	7.0 × 5.0 × 1.0 cm, raised nodular. Posterior wall previous anastamotic site	Poorly differentiated. Infiltration to the superficial muscularis. No lymph nodes identified	2 days postoperation CVA multiple (bilateral frontal lobes). Alive and NTR

NTR = no tumor recurrence; CVA, cerebrovascular accident.

In the other two cases, most of the tumor was located in the mucosa and submucosa, with superficial invasion of the muscularis. All perigastric lymph nodes submitted with the specimens were free of malignancy.

The malignant cells contained one or two prominent nucleoli within an oval nuclei with a clear to vesicular chromatin pattern. The eosinophilic cytoplasm was scant, with poorly defined borders. The tumor cells stained positively for keratin (Fig. 5), as detected with AE3, which identified their epithelial nature. They were weakly positive with the antikeratin AE1 and moderately positive for CEA. In all cases, the lymphocytic infiltrate was composed of small to medium-sized lymphocytes, with frequent plasma cells and occasional eosinophils. Most of the lymphocytes were T cells, as shown by the UCHL-1 stain (Fig. 6), and there were scattered B cells (L-26 positive). In two cases, B cells formed nodules with secondary follicles.

In Situ *Hybridization*

Sections incubated with the EBV-DNA probe by *in situ* hybridization yielded equivocal results.

PCR Results

Method 1. Southern blot results demonstrated replication products of EBV-DNA, as shown in Figure 7. Strong amplification products of EBV-DNA were present in cases 2 and 3, and amplification was less intense in case 1.

Method 2. Polymerase chain reaction amplification of DNA from paraffin sections showed a 110–base pair segment of DNA that was visible on ethidium bromide-stained gels. As shown in Figure 8, this segment annealed with the oligonucleotide probe that is specific for this region of the long internal direct repeat of the EBV genome, confirming that it was amplified from viral templates. A strong signal was evident in a one-hour autoradiogram, indicating a relative abundance of EBV genomes. Similar exposures of parallel amplification products from three

FIG. 1. Cross-section of a gastric carcinoma through the tumor in case 3, showing a plaque-like tumor extending to the muscularis propria.

FIG. 2. Low-magnification photomicrographs of the gastric tumor in case 1, showing a diffuse mononuclear cell infiltrate. Scattered large atypical tumor cells are seen singly or in clusters. Hematoxylin and eosin (×200).

conventional gastric adenocarcinomas did not yield a similar signal after one hour of exposure. A very faint signal, at the limits of discrimination, could be discerned when the exposure time was extended to 24 hours.

DISCUSSION

We have described three cases of primary gastric carcinoma with histologic features that are similar to those of nasopharyngeal LE. A review of all cases of gastric carcinoma seen from 1979 to 1989 (41 cases) at our medical center indicated no LEs other than those documented here. Recent reports identified LE-like carcinomas in the skin,[11] lung,[12] thymus,[13] salivary gland,[14] and uterine cervix.[15] To varying degrees, these have been poorly differentiated carcinomas composed of dispersed or clustered cells, with a dense lymphocytic infiltrate. The malignant cells in each of our cases stained positively for keratin. The lymphocytic background was a mixture of T and B cells, with the former predominating.

An association between nasopharyngeal LE and EBV has been documented. Nonetheless, most LE-like carcinomas outside the nasopharynx have not had easily demonstrable EBV genome segments by *in situ* hybridization.[10] Three of our cases had available paraffin blocks, and a DNA probe was used to detect the presence of the EBV genome. All cases were negative with the *in situ* hybridization technique, but they were positive with the PCR using two different methods aimed at two independent, highly conserved segments of the EBV genome.

These findings underscore the need for techniques that are more sensitive than *in situ* hybridization—namely, the PCR—to detect low numbers of DNA copies in LE cells.

The overall morphologic appearance of LE-like carcinomas of the stomach was unique among gastric tumors. Despite their relatively large size, each lesion was superficial, and pushing margins were seen grossly and microscopically. The similarity among these three cases was striking. The presence of EBV viral genome by PCR in all of the neoplasms confirms the findings of Burke and associates, who reported EBV genomes in one LE-like gastric carcinoma seen in a 71-year-old Filipino man. The latter tumor was histologically similar to those of our three patients.

Gastric carcinomas may assume several histopathologic appearances,[19,20] and some have been shown to have a rich lymphoid stroma.[21,22] Watanabe et al.[24] and Lertprasertsuke et al.[23] have focused on the varying intensity of lymphoid infiltration in relation to prognosis, rather than on possible epidemiologic factors. Inflammatory cell infiltrates in tumor stroma (particularly lymphocytic) have been considered for a long time to be a favorable prognostic factor in various neoplasms.

In a series of 1,041 gastric carcinomas, Watanabe and associates[24] found 24 cases (4%) in which a lymphoid stroma was pronounced; this infiltrate was interpreted as a host response. A high survival rate was found among the patients with this type of carcinoma. These authors emphasized the histomorphologic appearance of such le-
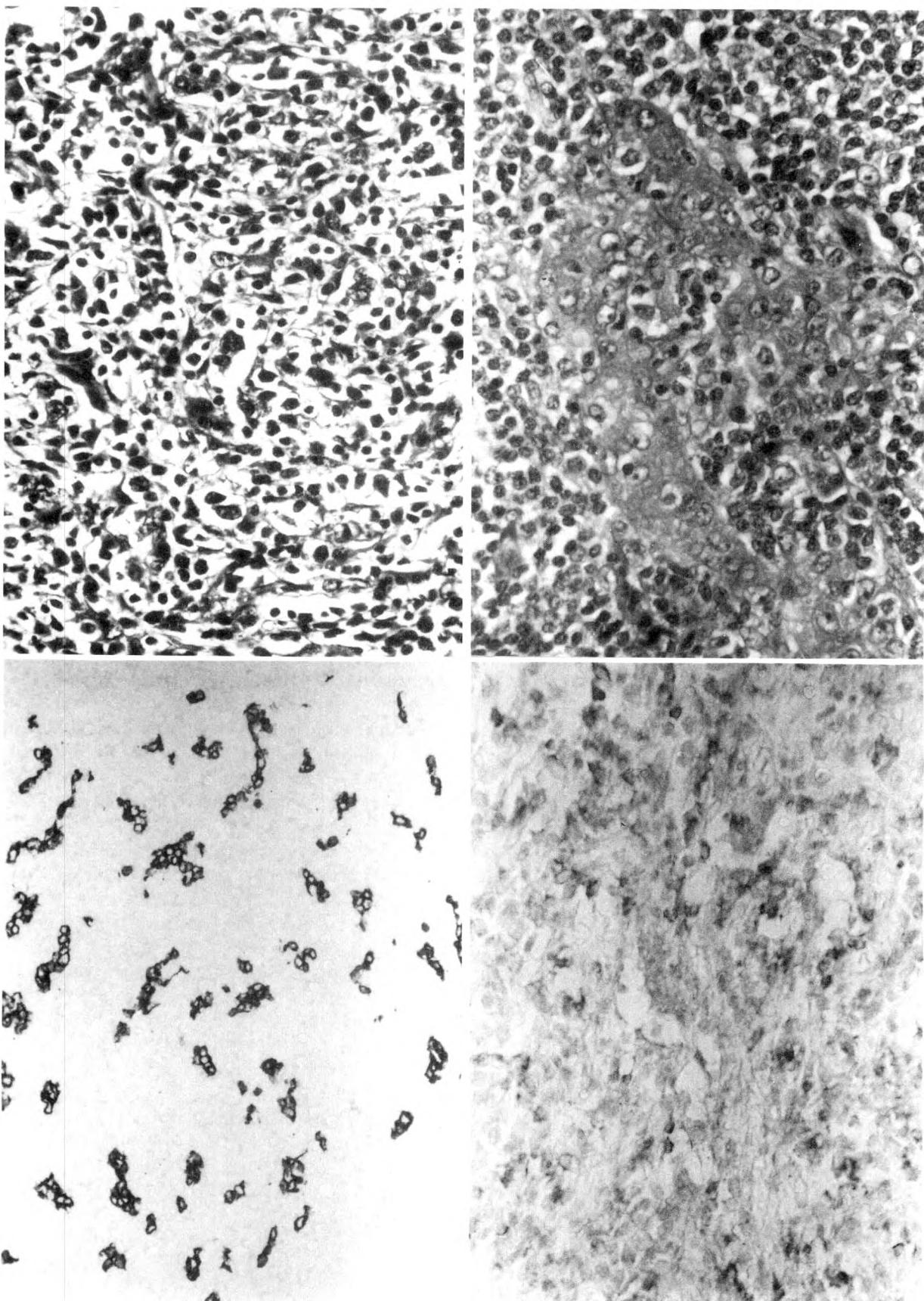
FIG. 3 (*upper, left*). Areas in some tumor in which undifferentiated tumor cells that formed syncitia. Hematoxylin and eosin (×300).

FIG. 4 (*upper, right*). The gastric tumor in case 2 showed rounded margins of infiltration. Note the pushing border of the tumor eroding into the muscularis. Hematoxylin and eosin (×5).

FIG. 5 (*lower, left*). Tumor cells showed strong positivity for cytokeratin. Avidin–biotin complex method, AE3 (×40).

FIG. 6 (*lower, right*). Lymphoid cells in the stroma were predominantly of the T-cell phenotype. Avidin–biotin complex method, UCHL (×200).

←

sions and suggested that they be designated "gastric carcinomas with lymphoid stroma" (GCLS). Subsequently, Lertprasertsuke and Tsutsumi[23] modified Watanabe's criteria; they found 24 cases of GCLS among 626 cases of gastric carcinoma, for an incidence of 3.8%.

While it is not clear whether the cases designated as GCLS by Watanabe and colleagues[24] and Lertprasertsuke and Tsutsumi[23] are similar to the neoplasms reported in this article, at least some of the cases included in the two series just cited seem to be similar to ours. Lymphoid stroma dominated the histologic appearance, as seen in nasopharyngeal LE.

The histologic features of the cases we have reported are similar to those of LE of other organs and represent

a unique subtype of gastric carcinoma. It should be noted that LE in the nasopharynx and sites outside it derive from the squamous epithelium, representing poorly differentiated squamous cell carcinomas. However, LE-like carcinomas of the stomach in our patients and the case reported by Burke and co-workers[16] derived from the glandular epithelium of the stomach. They represent poorly differentiated adenocarcinomas, as demonstrated by focal glandular formation, as seen in case 1. Suggestive gland formation was also noted in the illustration of the report by Burke and co-workers.[16] EBV genome also was identified by the PCR in a case of *in situ* adenocarcinoma of the cervix with heavy lymphoid stroma in a 52-year-old woman (unpublished personal observation). An as-
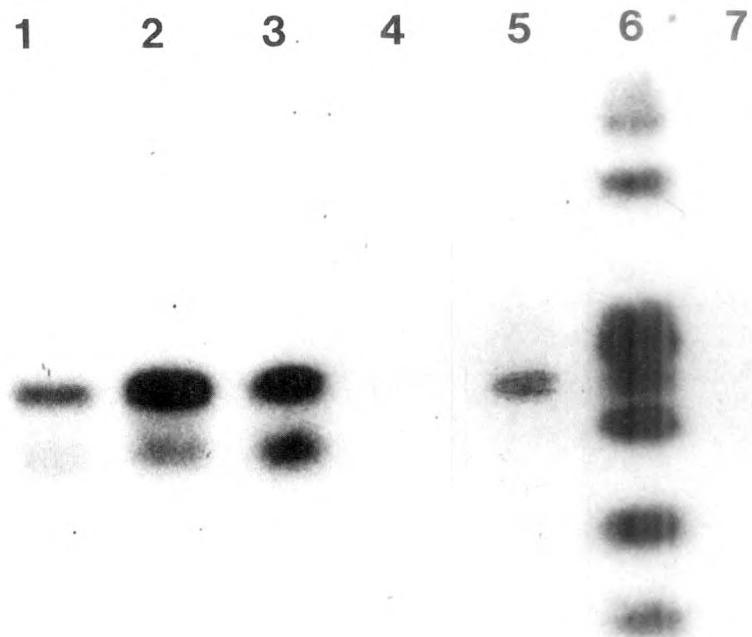

FIG. 7. Southern blot demonstrating amplified EBV-DNA by method 1. *Lane 1* = case 1, *Lane 2* = case 2; *Lane 3* = case 3; *Lane 4* = blank; *Lane 5* = Raji cell control; *Lane 6* = molecular weight markers; *Lane 7* = no DNA control.

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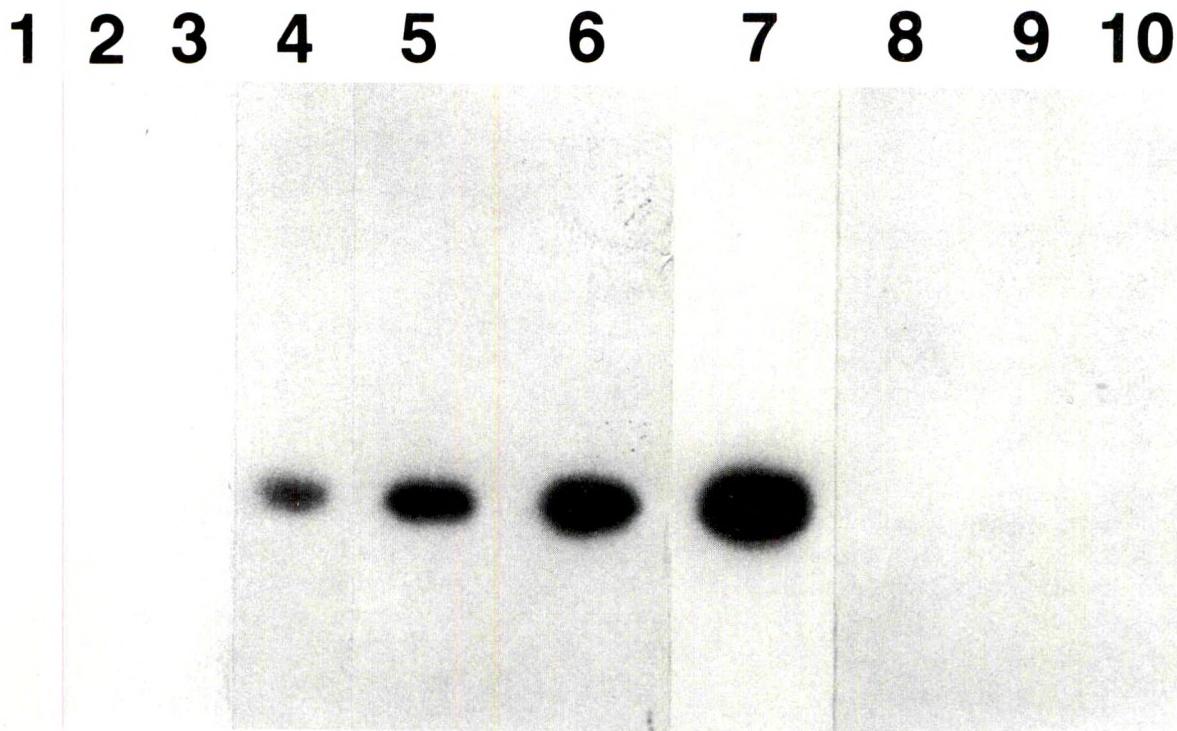
FIG. 8. PCR amplication of EBV genome by method 2. *1.* Normal human placental DNA; *2.* No DNA template; *3.* Buffer; *4.* Raji cell DNA; *5.* case 3; *6.* case 2; *7.* case 1; *8.* GCOH1; *9.* GCOH2; *10.* GCOH3. GCOH = gastric carcinoma of ordinary histology.

sociation of the EBV with uterine cervical adenocarcinoma also was suggested by Singh and co-workers.[25] They reported that three Chinese women developed uterine cervical adenocarcinomas following the diagnosis of nasopharyngeal LE.

Thus, it seems reasonable to conclude that EBV not only is associated with poorly differentiated squamous cell carcinoma with lymphoid stroma (so called LE) but also with poorly differentiated adenocarcinomas with lymphoid infiltration. The presence of EBV-DNA in these tumors also supports the claim that LE-like carcinomas of extrapharyngeal organs are similar to nasopharyngeal tumors.

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Multitumor "Sausage" Blocks in Immunohistochemistry

Simplified Method of Preparation, Practical Uses, and Roles in Quality Assurance

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This report describes a simplified method for preparing multitumor sausage blocks (MTSBs) for use in immunohistochemical procedures. Rather than relying on previously processed paraffin blocks as a source of material, this procedure involves procuring tissue at the time of gross specimen examination. The tissue is processed along with routine surgical pathologic material, and the paraffinized samples are placed in "storage cassettes" for easy cataloging and storage. Thin strips are cut from the tissue in the "storage cassettes" and combined by dripping liquid paraffin onto them while they are rolled between the thumbs and forefingers, somewhat like making a cigarette. This results in a tissue "log." Transverse sections of the "log" are embedded in paraffin blocks and used as MTSBs. Practical uses of MTSBs are discussed, and their role in quality assurance is stressed. (Key words: Multitumor sausage blocks; Quality assurance; Immunohistochemistry; Immunoperoxidase) Am J Clin Pathol 1991;96:228–232

In 1986, Battifora described a method for preparing multitumor sausage blocks (MTSBs), which allowed several different pieces of tissue to be placed compactly in a single paraffin block.[1] With the use of this technique, tissue from many types of tumors could be immunostained using a single MTSB slide and, hence, with only a single drop of antibody. Battifora demonstrated that the use of MTSB slides was an efficient and cost-effective way of screening for the specificity of monoclonal antibodies and monitoring the sensitivity of immunohistochemical procedures.

In this report, a simplified method of MTSB preparation is described, and practical uses of MTSBs are discussed, including their use in quality assurance pertaining to immunohistochemical procedures.

MATERIALS AND METHODS

Fresh tissue was obtained at the time of gross examination of various tumors, and tumor tissue was cut into rectangular blocks (0.2–0.3 cm thick × 2 cm × 2.5 cm) if sufficient tumor was available. If tissue was limited in

amount, strips of tissue measuring 0.2–0.3 cm on a side × 2–2.5 cm long were used. These tissue blocks or strips were placed in plastic tissue cassettes (labeled with the tumor type and accession number), fixed in formalin (or other fixative as desired), and processed along with routine surgical pathology material in a tissue processor (Miles VIP®, Miles Inc., Diagnostic Division, Mishawaka, IN). These tissue cassettes (called "storage cassettes") were removed from the final liquid paraffin bath of the tissue processor, and the paraffin was allowed to drain from the cassettes. They were cooled to room temperature, and storage cassettes containing the paraffinized tissue were stored and cataloged (Fig. 1).

When an MTSB was being made, a variety of tumor types were selected from the appropriate storage cassettes, and thin strips (0.1 cm in diameter × 2.5–3 cm long) were removed from the paraffinized storage tissue with the use of a razor blade (Fig. 2). (A "key" should be kept of the various types of tumors selected for inclusion in each MTSB, and the types should be separated into groups [Fig. 3]). With the operator wearing rubber gloves at a tissue-embedding center, liquid paraffin was dripped onto a group of thin strips of tumor tissue, while the strips were rolled between the thumbs and forefingers, somewhat like making a cigarette (Fig. 4). As the paraffin cooled, it bound the tissue strips, and they could be compressed and worked into a compact roll while the paraffin congealed. More

Multitumor "Sausage" Blocks

SAUSAGE BLOCK #6

| Melanoma | S90-3005 |
	S-38-88
	S-7229-87
	S-3017-87
	S89-4189
Gliosis	S89-2649
Astrocytoma	S90-584
Granular cell tumor	S89-2868
Meningioma	S8872-87
	S7469-88
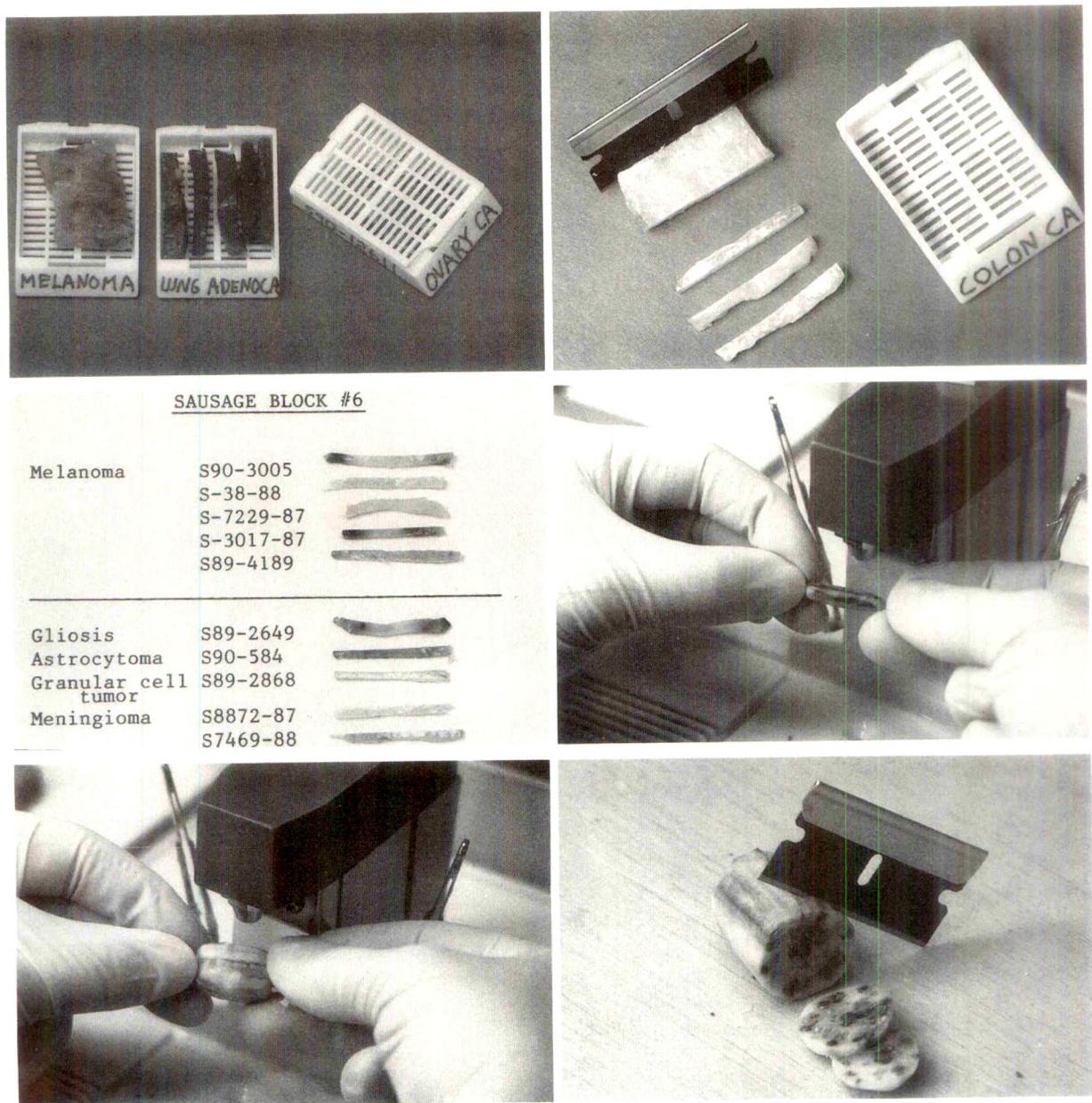
FIG. 1 (*upper, left*). Paraffinized pieces of tumor tissue are placed in labeled tissue cassettes (*storage cassettes*). These "storage cassettes" are stored and cataloged easily.

FIG. 2 (*upper, right*). The first step in preparing an MTSB involves slicing thin strips of tissue from the paraffinized "storage" tissue with the use of a razor blade.

FIG. 3 (*center, left*). Example of "key" used to record types of tumor and case numbers used in preparation of an MTSB.

FIG. 4 (*center, right*). While strips of tissue are held between the thumbs and forefingers at a tissue-embedding center, liquid paraffin is dripped onto the tissue while it is rolled between the thumbs and forefingers, like making a cigarette. As the paraffin cools, it binds the tissue strips.

FIG. 5 (*lower, left*). Adding additional tissue strips or groups of tissue strips is performed easily by repeating the procedure illustrated in Figure 4, resulting eventually in a tissue "log" containing numerous strips of tumor tissue embedded in and bound together by paraffin.

FIG. 6 (*lower, right*). The tissue "log" is cut transversely at 0.3-cm intervals, providing pieces for use as MTSBs. It is easiest to cut these transverse sections while the "log" is still warm.

strips were added easily to the roll by repeating the above process (Fig. 5).

When the desired number and types of tumors had been added to the roll, the resulting tissue "log" was allowed to cool, and 0.3-cm serial transverse sections were cut from the "log" (Figs. 6 and 7). The transverse sections were taken while the "log" was somewhat warm because it could be cut more easily and maintained its structural integrity best. These transverse sections of the tissue "log" then were embedded in paraffin blocks and used as MTSBs (Fig. 8).

In his article, Battifora[1] described a method for subdividing the MTSB by wrapping small groups of tissue strips in sausage casing.[1] Similar subdivisions also were made with the use of the simplified MTSB method. Because sausage casing was difficult to procure in our metropolitan area, amnionic membrane stripped from the surface of a placenta was used as a suitable alternative. To prepare the amnion for use in making MTSBs, first it was stripped from the fetal surface of the placenta and wrapped around a thin wooden stick (0.2-cm diameter, wooden-handled cotton swabs). This "jelly roll" of amnion was fixed in formalin and placed in a tissue processor (Miles VIP) along with routine surgical pathologic material. The amnion roll then was removed from the final liquid paraffin bath of the tissue processor and unrolled while still warm. The excess paraffin was allowed to drain, and the paraffin-embedded amnionic tissue was cooled and stored at room temperature (Fig. 9).

The paraffinized amnionic tissue was used to wrap subgroups of tumor tissue strips; to accomplish this, small segments were cut from the stored amnionic tissue (3 cm × 2 cm), the segments were heated on the block of a tissue-embedding center, and the amnion was wrapped around the desired group of tissue strips (Fig. 10). As the amnion cooled, it bound well to the tissue strips, and the resulting wrapped group of tumor strips was added easily to similar subgroups, with the use of the method described above. In this fashion, a number of "minisausages" were combined into a larger "sausage" for use as an MTSB (Fig. 8).

RESULTS

With the methods described above, MTSBs of any desired composition were prepared easily. The MTSBs then were cut at 5 μm and mounted at one end of a microscopic slide for use in immunohistochemical staining procedures. We used MTSB sections for positive control tissue sections; mounting the MTSB section on one end of the slide allowed additional tissue (*e.g.*, a diagnostic case to be studied) to be placed on the same slide, decreasing the number of sections that needed to be manipulated. Several examples of sausage block slides are illustrated in Figure 11.

DISCUSSION

In 1986, Battifora described his method of MTSB preparation and suggested it as a novel and efficient method of screening monoclonal antibodies and monitoring the sensitivity of immunohistochemical studies. We have used Battifora's method for preparing MTSBs but have encountered a number of difficulties, including an inability to procure sausage casing from local food stores or meat markets. Moreover, we found that Battifora's method was somewhat cumbersome and complicated. Because of these problems, we believe that his method probably will not be used by many practicing pathologists.

The procedure that we have described is simple to use and provides the same end result as Battifora's method. Our technique also ensures that the tissue in the MTSB is processed in an identical fashion to the laboratory's routine surgical pathologic material, making it ideal control material. MTSB sections can be obtained commercially (Xenetics Biomedical, Inc., Tustin, CA), but they are prohibitively expensive and involve the problematic issue of human tissue sales. Moreover, they may be processed differently from the routine material in one's own laboratory.

Since Battifora's original description of MTSBs, several other methods of preparing similar material have been reported. One of these involves procurement of tissue from paraffin blocks by punching cores of tissue from them with the use of a 16-gauge hollow needle and placing the cores into drinking straws before they are embedded in paraffin.[2] Another method uses a skin-punch biopsy instrument to remove tissue from paraffin blocks, and the cores then are combined in a single paraffin block for use as an MTSB.[3] Both of these methods are suitable alternatives. However, with the procedure we describe, removal of material from paraffin blocks is avoided and many more MTSB sections can be obtained from a single preparation because a tissue "log" can yield six or seven MTSBs (rather than a single MTSB with other "second-generation" methods). As such, our technique offers distinct advantages if MTSB sections are to be used in high volumes, as they are in our laboratory.

Quality assurance of immunohistochemical procedures is an important issue,[4–7] and MTSBs can have an important role in the maintenance of quality control in diagnostic immunohistochemistry. In our laboratory, we use MTSB slides for all positive control tissue sections. With appropriate selection of the component tissues of the MTSB, a single block can be used as a positive control
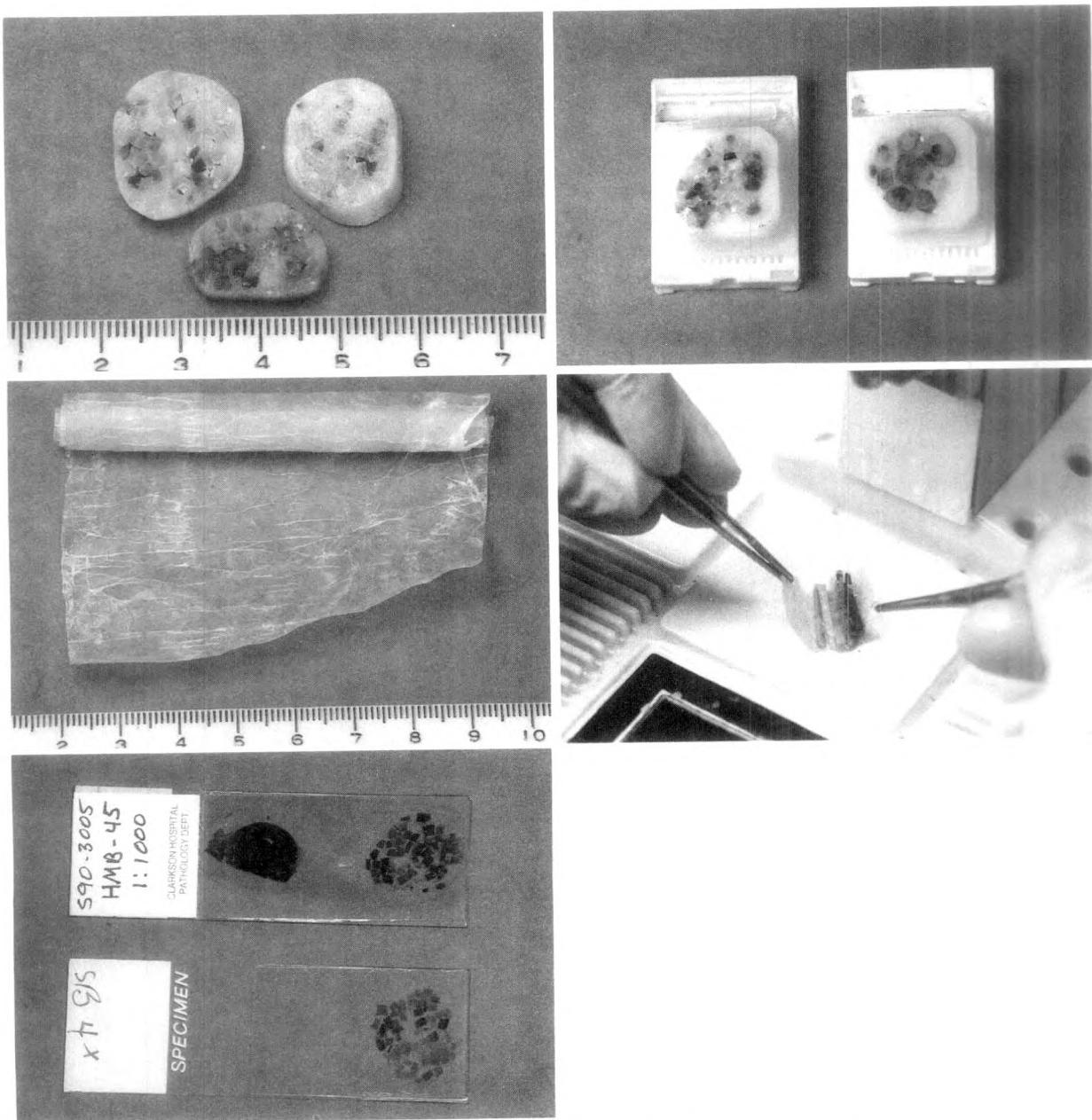
FIG. 7 (*upper, left*). Typical cross-sections taken of a tissue "log," ready to be embedded in paraffin blocks and used as MTSBs.

FIG. 8 (*upper, right*). Completed MTSB, ready for sectioning.

FIG. 9 (*center, left*). Photograph of paraffinized roll of amnionic membrane that can be cut into strips and used to subdivide MTSBs.

FIG. 10 (*center, right*). Group of tumor tissue strips being wrapped in paraffinized amnion (on the heating block of an embedding center) to subdivide them from other strips to be placed in the MTSB.

FIG. 11 (*lower*). Typical sections cut from an MTSB. Note that the MTSB section is mounted toward one end of the slide, leaving additional space for a section of other tissue to be mounted on the opposite end of the slide. This can substantially reduce the number of slides being manipulated by technologists performing the staining.

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for essentially any stain that may be desired. This greatly simplifies demands on histotechnologists and obviates the need for a different positive control section with each primary antibody that is used. In addition, MTSB slides are superior to single-tissue positive control sections because one can assess a number of different positive constituent tumor types, as well as neoplasms in the MTSB that should *not* stain.

When the MTSB sections are mounted at one end of a microscopic slide, large numbers of MTSB sections may be precut, mounted, and stored for use as controls. When tissue for a diagnostic case is to be stained, it can be placed at the other end of the precut MTSB slide, so that the test tissue and the positive control MTSB section are present on a single slide (Fig. 11). This almost halves the number of slides that need to be manipulated by the technologist. Because an MTSB section is always present on the same slide as the test tissue, it is a consistent monitor of sensitivity and specificity for any given stain that is performed for diagnostic purposes. This can be invaluable if a question arises regarding the performance of a particular antibody. One needs only to view the MTSB section on the same slide to quickly assess the specificity and sensitivity of the stain in a given case.

Another major use of MTSB sections (mentioned only rarely by previous authors[3]) is their applicability in determining the optimal titers of primary antibodies. In our experience, suboptimal reagent dilutions are a common cause of poor or inappropriate staining, particularly if laboratories blindly follow the manufacturers' recommendations for titers or prediluted antibodies are used unquestioningly as supplied. Our experience (unpublished) has shown that reliance on the suggestions of a manufacturer without evaluation of additional "in-house" titers invites disaster. On numerous occasions, we have found that recommended antibody dilutions are unacceptable, and the difference between suggested titers and optimal titers has varied by a factor as high as 256 (*i.e.*, a "ready-to-use" prediluted antibody must be diluted by a factor of 1:256 before appropriate staining is observed). Using MTSB sections to determine proper antibody titers allows one to confirm that known negative cases are indeed nonreactive (*e.g.*, that a known lymphoma does not

label with HMB-45). This is just as important as assuring oneself that known positive cases are reacting as expected. Also, one easily can become familiar with the spectrum of reactivity of the antibodies being used. The latter is an important point because manufacturers' claims regarding sensitivity and specificity may not always (and indeed should not) be trusted.

In conclusion, the simplified method of MTSB preparation allows any hospital laboratory to use these sections in their diagnostic immunohistochemistry service, using in-house material. By selecting the components of the MTSB appropriately, one can greatly simplify the problem of procuring positive control tissue sections because a single MTSB then can be used as a positive control section for many different primary antibodies. MTSB sections can assume a major role in quality assurance in diagnostic immunohistochemistry by repetitively documenting the sensitivity and specificity of antibodies that are used in any case that is studied. Lastly, MTSB sections are ideal for the determination of optimal titers of antibody reagents.

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Original Article

Potential Use of Monoclonal Antibodies in the Diagnostic Distinction of Gynecomastia from Breast Carcinoma in Men

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Immunohistochemical (IHC) assays using the monoclonal antibodies (MoAbs) B72.3 and B6.2, recognizing two distinct and independently expressed breast tumor–associated antigens (BTAAs), recently have been shown to significantly improve the accuracy of cytodiagnosis of breast nodules by fine-needle aspiration (FNA). To evaluate whether the same method may be useful diagnostically in distinguishing gynecomastia from breast cancer in men, a retrospective avidin-biotin immunoperoxidase assay study was performed on 50 cases of gynecomastia and 30 cases of breast carcinoma in men, using a panel of five MoAbs known to recognize different BTAAs. The results of this study demonstrated that MoAbs B1.1, HMFG2, and MBr1 displayed a strong reactivity with gynecomastia and carcinoma, but MoAbs B72.3 and B6.2 separated benign and malignant lesions in a high percentage of cases. When used in combination, the latter two reagents reacted with 96% of the carcinomas that were analyzed but labeled only 67% of gynecomastia cases. Thus, the conjoint use of these two reagents may enhance the use of FNA biopsy as a valuable tool in the presurgical diagnosis of breast nodules in men. (Key words: Breast carcinoma; Gynecomastia; Monoclonal antibodies; Fine-needle aspiration) Am J Clin Pathol 1991; 96:233–237

Carcinoma occurs infrequently in the male breast, and our knowledge of its biology and clinical behavior largely has been developed through a compilation of pooled experiences.[1,2] Altered hormonal metabolism and antecedent gynecomastia appear to play a role in the development of the disease.[3,4] Therefore, breast masses in men that are suspected to be malignant necessitate the differential diagnosis between gynecomastia and carcinoma.[2]

Fine-needle aspiration (FNA) cytology, which is applied widely in evaluating the nature of breast nodules in women, also could be a valuable tool in the clinical diagnosis of breast lesions in men. In most cases, conventional cytologic examination affords a definitive diagnosis, but the presence of dyscohesive, hyperchromatic cells in FNA from gynecomastia can mimic carcinoma and cause diagnostic confusion.[5,6]

We have demonstrated recently that the use of selected panels of monoclonal antibodies (MoAbs) to tumor-associated antigens (TAAs) can be a useful complement to conventional cytopathology[7,8] and significantly improve the diagnostic accuracy of FNA of breast masses in women.[9,10] To evaluate whether the same method could be helpful diagnostically in distinguishing gynecomastia from breast carcinoma in men, we analyzed the antigenic phenotype of 50 cases of gynecomastia and 30 cases of breast carcinoma in men, using a panel of five MoAbs that recognize distinct breast TAA (BTAA). The results of this retrospective study demonstrated that MoAbs B72.3 and B6.2 separated benign from malignant breast lesions in men in a high percentage of cases. Thus, these monoclonal antibodies may be useful tools in the presurgical cytodiagnosis of these rare carcinomas in men.

MATERIALS AND METHODS

Tissue Samples

Formalin-fixed, paraffin-embedded tissues from 50 cases of gynecomastia (mean patient age, 48.5 years; range, 16–74 years) and 30 breast carcinomas from men (mean patient age, 59.1 years; range, 23–77 years) were obtained from the Departments of Pathology at the Regina Elena

Cancer Institute and the Catholic University of Rome. The breast tumors from men represented surgical biopsy specimens collected from 1978 to 1989. Five-micron sections from each block were mounted on gelatin-coated slides. Hematoxylin and eosin–stained sections were reviewed to confirm previous histologic diagnosis.

Fine-needle aspiration biopsies from six breast nodules were performed with the use of a 21-gauge needle and a 20-mL disposable syringe mounted on a special holder (Cameco® 20 mL; Precision Dynamics, Burbank, CA). Cell smears were fixed in cold acetone for 10 minutes and stored at −20 °C. Our prior experience showed that this procedure caused no loss of reactivity for at least four months.

Monoclonal Antibodies

Monoclonal antibodies B72.3[11,12] and B6.2[13] were purchased from Sorin Biomedica Company (Saluggia, Italy), and MoAb HMFG2[14,15] was obtained from Oxoid Company (Milano, Italy). MoAbs MBr1[16] and B1.1[17] were provided by Dr. M. I. Colnaghi (National Cancer Institute, Milano, Italy) and Dr. J. Schlom (Laboratory of Tumor Immunology and Biology, National Cancer Institute [National Institutes of Health], Bethesda, MD), respectively.

The immunoreactivities of these reagents were assessed repeatedly during the study, with the use of negative and positive control specimens. All MoAbs were used as purified reagents (B72.3 and B6.2) or as appropriately diluted ascites fluid preparations.

Immunoperoxidase Technique

The avidin–biotin peroxidase complex assay was performed on dewaxed, rehydrated, 5-μm sections of formalin-fixed, paraffin-embedded tissues or on FNA preparations with the use of a commercially available kit (IMMUCOLOR®, Sorin Biomedica). Slides were incubated with MoAbs to BTAA at 4 °C for at least 16 hours in a moist chamber. The protein concentration of the primary antibodies ranged from 25 to 50 μg/mL in Hanks' balanced salt solution (HBSS) containing 1% bovine serum albumin (BSA) (Sigma Chemical Company, St. Louis, MO). Bound peroxidase was visualized with the use of 3-amino-9-ethylcarbazole (AEC) as the chromogenic substrate, for 8 minutes at room temperature. Slides then were rinsed with phosphate-buffered saline (PBS) (0.15 mol/L) and counterstained with Mayer's hematoxylin (BDH Chemicals, Ltd., Pool, United Kingdom). Finally, the sections were mounted in buffered glycerol. Immunohistochemical reactivity was scored as "heterogeneous" when 25–50% of the specimen was stained and "homo-

geneous" when the reactivity involved more than 50% of the tumor cell population.

RESULTS

Monoclonal Antibodies

The MoAbs used in this study, with their relevant references, are listed in Table 1. They included five reagents that are known to recognize distinct BTAAs in women and were chosen on the basis of their ability to react with formalin-fixed, paraffin-embedded tissues. This characteristic permitted a retrospective analysis of gynecomastia and breast cancers in men.

Analysis of the Antigenic Phenotype of Gynecomastia and Breast Carcinoma in Men

Among the five MoAbs used in this study, B1.1, HMFG2, and MBr1 displayed intense reactivity with 86–100% of all cases of gynecomastia. MoAb B72.3 did not show detectable reactivity with benign lesions, and MoAb B6.2 reacted heterogeneously in only 3 of 50 cases (6%) of gynecomastia. The predominant staining pattern of MoAbs B1.1, HMFG2, and MBr1 was homogeneous and was restricted, in most instances, to the plasma membrane (Fig 1A).

Among 30 breast carcinomas from men that were tested with our panel of reagents, a homogeneous pattern of reactivity was observed in 80–90% of the malignant lesions with MoAbs B72.3 and B6.2 (Fig. 1B).

Monoclonal antibody B1.1 reacted with 86.6% of these cases, and all of the breast carcinomas from men showed intense and predominantly cytoplasmic reactivity with MoAbs HMFG2 and MBr1 (Table 2).

When immunocytochemical analysis was performed on six FNA specimens from two malignant and four benign mammary tumors from men, we observed the same staining pattern as that seen in paraffin sections (Table 3).

DISCUSSION

Cancer of the male breast is a rare disease, accounting for approximately 1% of all reported cases of mammary

TABLE 1. MONOCLONAL ANTIBODIES USED TO ANALYZE THE ANTIGENIC PHENOTYPE OF BREAST CARCINOMAS IN MEN

MoAbs	Isotype	Antigen	Molecular Weight	References
B1.1	IgG1	Glycoprotein	180×10^3 daltons	17
HMFG2	IgG1	Glycoprotein	220×10^3 daltons	14, 15
MBr1	IgM	Glycolipid	Unknown	16
B72.3	IgG1	Glycoprotein	$>10^6$ daltons	11, 12
B6.2	IgG1	Glycoprotein	90×10^3 daltons	13
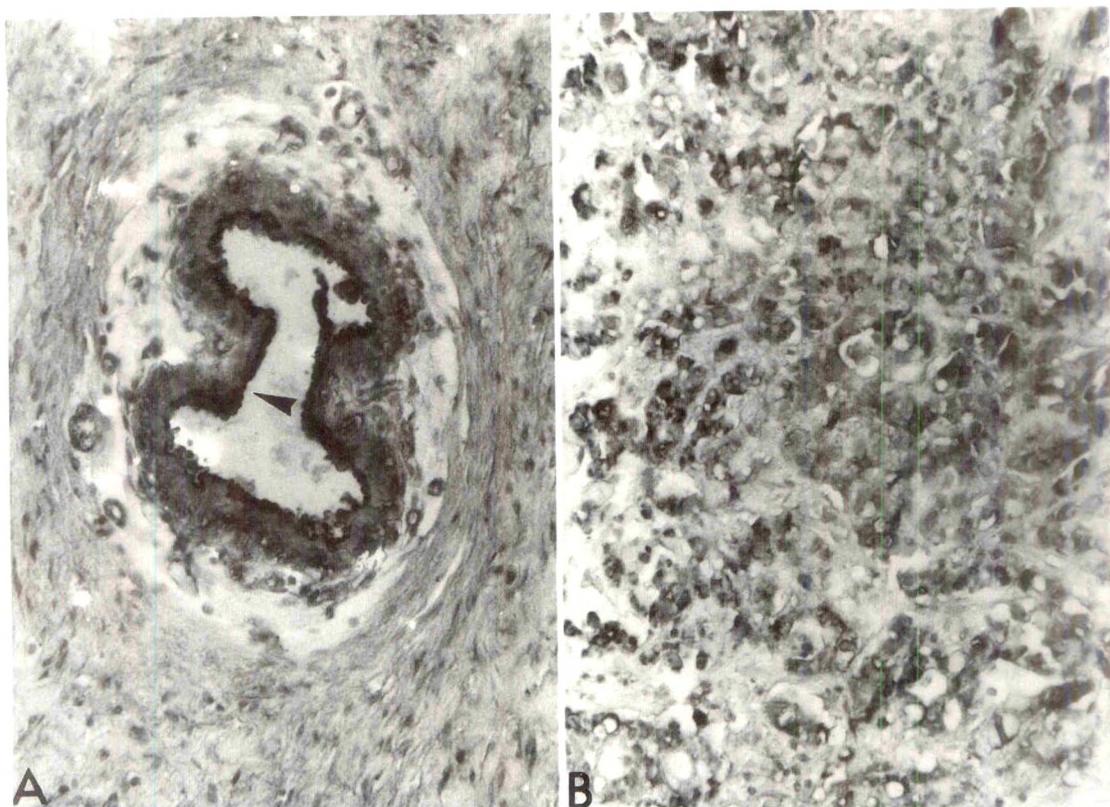
FIG. 1. *A.* Gynecomastia, showing homogeneous membranous cytoplasmic staining pattern with MoAb B1.1 (×200). *B.* Breast cancer from a man, showing intense cytoplasmic staining with MoAb B72.3 (×200). Mayer's hematoxylin counterstain.

carcinoma. In contrast, gynecomastia is a common condition, showing a variable age of presentation, with the greatest prevalence in men in the third decade of life.[2,3,18] Although the histopathologic features of the two lesions usually cause no diagnostic challenges, the clinical distinction between gynecomastia and breast carcinoma in men may be difficult. Presenting symptoms and signs may be identical in these conditions.[19,20]

In the current study, a retrospective avidin-biotin immunoperoxidase assay analysis was performed on 50 cases of gynecomastia and 30 examples of breast carcinoma

from men, using a panel of five MoAbs (B72.3, B6.2, HMFG2, MBr1, B1.1). These reagents previously have been shown to react with benign and malignant breast lesions in women.

Murine MoAbs B72.3 and B6.2 were generated by immunizing mice with membrane-enriched fractions of breast carcinoma metastases from the liver. The corresponding antigens appear to be expressed selectively by transformed female mammary epithelium in 75% and 80% of cases, respectively.[11-14] MoAb MBr1 recognizes a cell membranous neutral glycolipidic antigen that is ex-

TABLE 2. ANTIGENIC PHENOTYPE OF GYNECOMASTIA AND BREAST CARCINOMA IN MEN USING A PANEL OF MONOCLONAL ANTIBODIES TO DISTINCT FEMALE BREAST TUMOR-ASSOCIATED ANTIGENS

Histologic Diagnosis	Immunohistochemical Reactivity	Monoclonal Antibodies				
		B1.1	HMFG2	MBr1	B72.3	B6.2
Gynecomastia		*43/50 (86%)	50/50 (100%)	46/50 (92%)	0/50 (0%)	3/50 (6%)
	Heterogeneous†	10	12	12	0	3
	Homogeneous†	33	38	34	0	0
Carcinoma		*23/50 (85.5%)	30/30 (100%)	30/30 (100%)	24/30 (80%)	27/30 (90%)
	Heterogeneous‡	7	6	3	2	5
	Homogeneous‡	19	24	27	22	22

* Fraction positive. Subjacent column entries show number of cases with each staining pattern.   Staining was †apical or ‡cytoplasmic.

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TABLE 3. IMMUNOCYTOCHEMICAL ANALYSIS OF FINE-NEEDLE ASPIRATES OF BREAST NODULES IN MEN USING A PANEL OF MONOCLONAL ANTIBODIES RECOGNIZING FEMALE BREAST TUMOR-ASSOCIATED ANTIGENS

Condition	No. of Cases	*Immunocytochemical Reactivity*				
		B1.1	*HMFG2*	*MBr1*	*B72.3*	*B6.2*
Gynecomastia	4	3/4*	4/4	4/4	0/4	0/4
Carcinoma	2	1/2*	2/2	2/2	1/2	2/2

* Fraction positive.

pressed by normal epithelium and roughly 80% of carcinomas of the breast.[16] The MoAb HMFG2, produced by immunizing mice with human milk fat globule membrane and cultured human milk-producing cells, labels a group of high molecular weight (HMW) mucin glycoproteins expressed by more than 80% of normal breasts and benign mammary lesions and 95% of breast carcinomas.[14,15] MoAb B1.1 precipitates iodinated carcinoembryonic antigen, resulting in a radiolabeled peak at approximately 180 kD. This reagent reacted with 66% of the female mammary carcinomas that were tested.[17]

This IHC evaluation was conducted so that those reagents that might be useful in the cytologic diagnosis of breast masses in men could be selected.

Fine-needle aspiration biopsy has been used increasingly in the presurgical assessment of breast cancer in women, whereas gynecomastia and mammary carcinoma in men have undergone only limited analysis with this diagnostic procedure.[6,21,22] This is unfortunate, because aspirates from lesions of gynecomastia sometimes may be misinterpreted as malignant because of their cellularity, dyscohesion, and anisonucleosis.[5,6,23]

In previous studies, we have demonstrated that MoAbs B72.3 and B6.2, when used in combination, can significantly improve the diagnostic accuracy of FNA of female breast nodules[10] and diminish false-negative interpretations. These findings were confirmed recently in a multicenter study.[9]

In the current series, we demonstrated that MoAbs B1.1, MBr1, and HMFG2 showed an intense pattern of reactivity in benign and malignant breast nodules in men, as described in breast lesions in women.[15–17] Eighty percent to 100% of gynecomastia cases expressed BTAAs that were recognized by these three reagents, with a staining distribution that was associated with plasma membranes. When tested with the same MoAbs, breast cancers from men displayed a comparable percentage of reactivity, but with a more homogeneous and cytoplasmic staining pattern. The combination of MoAbs B72.3 and B6.2, which recognize BTAAs that are expressed independently by transformed cells, distinguished between benign and malignant lesions in men in 96% of cases. The same pattern

of reactivity also was observed in a few FNA specimens from tumors of the male breast that were analyzed with avidin–biotin peroxidase complex assays.

These findings show that breast cancers in men and women have significant clinical and morphologic similarities[19] and also share a comparable antigenic phenotype, as previously described by other authors using different MoAbs.[24] However, to date, no study has provided information on the patterns of antibody reactivity in gynecomastia that would link it with carcinoma. This would be of clinical interest, because gynecomastia has been suggested as a condition predisposing men to breast cancer.[1,2] Although clinical gynecomastia rarely is associated with breast carcinoma in general terms, microscopic features of the former lesion have been described in as many as 40% of men with breast cancer.[19,20] This apparent discrepancy can be explained simply, by focusing on the wide difference in the prevalence of these two diseases.

Of additional interest is the shared expression of the antigens identified by MoAbs B72.3 and B6.2 in malignant mammary epithelium in both women and men. These data may indicate that common pathogenetic mechanisms are operative in men and women with breast cancers, despite the origins of these neoplasms in different metabolic (hormonal) milieus. From a practical point of view, the expression of similar macromolecules in such tumors offers an objective criterion for the separation of gynecomastia from cancer of the male breast. If this IHC observation is confirmed in a larger series of cases, the use of FNA biopsy in the presurgical diagnosis of breast cancer in men may be applied more effectively.

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Original Article

Expression of Haptoglobin-related Protein in Primary and Metastatic Breast Cancers

A Longitudinal Study of 48 Fatal Tumors

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The ability to establish a prognosis for patients with early breast cancer is an important clinical issue. Recent studies have shown that antibodies to haptoglobin-related protein (Hpr) may be useful in stratifying early patients with breast cancer according to their relative risks of recurrence. Nearly 30% of early breast cancers express proteins bearing Hpr epitopes. Hpr-positive breast cancers are more likely to recur after primary resection and are associated with shorter disease-free intervals. This immunohistochemical study examines temporal changes in Hpr expression during the course of disease in 48 patients with fatal breast carcinoma. Thirty-seven primary tumors (77%) were Hpr positive. Ten of the 11 initially negative tumors (91%) were Hpr positive at the time of recurrence. In contrast, only 10 of the 37 initially positive tumors (27%) were Hpr negative with relapse. Of 18 axillary nodes that were examined, 16 (89%) were Hpr positive; all four lymph nodal metastases in patients with initially negative primary tumors were Hpr positive. The authors conclude that the acquisition of Hpr expression parallels increased malignant potential and that Hpr expression, once acquired, tends to remain a permanent characteristic of any given mammary tumor. (Key words: Breast carcinoma; Haptoglobin-related protein; Prognosis; Autopsy; Immunohistochemistry; Monoclonal antibodies) Am J Clin Pathol 1991;96:238–242

Some human breast carcinomas express the haptoglobin-related protein (Hpr) or proteins that share epitopes with it.[1,2] In a recent retrospective immunohistochemical study of 70 patients with early breast carcinoma (stages I and II), Kuhajda and associates[2] showed that Hpr epitope expression by mammary tumors was associated with a significantly increased risk of recurrence. These studies suggested that a link exists between Hpr-epitope expression and the development of aggressive malignant potential. Therefore, if this phenomenon represents a biologic event linked to the heightened expression of malignancy,

it is expected to occur in metastasis as the disease progresses. Moreover, metastases from initially Hpr-negative tumors would be expected to acquire Hpr epitopes.

The current study evaluated the temporal changes in Hpr epitope expression over the course of disease in cases of fatal breast carcinoma.

MATERIALS AND METHODS

Case Selection

The autopsy records were reviewed of patients who died of breast carcinoma at the Johns Hopkins Hospital between 1960 and 1988. The most recent 48 consecutive cases for which slides and paraffin blocks were available from the primary tumor, the autopsy material, or any intervening recurrences were selected for this study.

Evaluation of Clinical and Pathologic Features

All available relevant clinical case summaries and pathology records were reviewed and abstracted. Slides from the primary tumor were evaluated to determine the type of tumor and its extent. Representative blocks of the pri-

mary neoplasm, and, whenever possible, its lymph node metastases, were selected for immunohistochemical studies. Slides from the recurrences and autopsy material were examined to verify the presence of tumor and to allow a representative block to be selected for immunohistochemical staining.

Preparation of Anti-Hpr Antibodies

A polyclonal antibody was produced by immunizing New Zealand white rabbits with a synthetic peptide corresponding to the 34 N-terminal residues of the predicted Hpr gene product and affinity-purifying the resulting serum as described previously.[1,2] This antibody was used in the preliminary phases of the study and later was replaced with a monoclonal anti-Hpr, a murine IgG2b kappa.

Monoclonal antibodies were prepared according to a modification of the method described by Zola.[3] Three BALB/c mice were immunized with the use of an Hpr-derived synthetic peptide-keyhole-limpet hemocyanin conjugate,[1,2] and their spleens were fused with plasmacytes from the SP2 murine myeloma cell line. Hybridoma supernates initially were screened by enzyme-linked immunosorbent assay (ELISA), with the use of the synthetic peptide conjugated to bovine serum albumin as the antigen, and positive wells were expanded and cloned by limited dilution. Clones were rescreened with ELISA. Those that were positive were characterized further with the use of Western blots with haptoglobin 1:1 and 2:2, and an Hpr preparation that was obtained by a method described previously.[1] Only antibodies that reacted with tumor sections that were known to be positive with the polyclonal antiserum were selected. The monoclonal antibody was purified from ascites fluid with the use of protein A–Sepharose® affinity chromatography (Sigma Chemical Co., St. Louis, MO) with elution by 6 mol/L urea in phosphate-buffered saline (PBS). The eluate was collected and dialyzed against PBS overnight in cold temperatures; then it was aliquoted and stored frozen at -80 °C, or lyophilized.

Immunohistochemistry

Immunohistochemical studies were performed with the use of the unlabeled antibody–immunoperoxidase technique.[4] We evaluated routinely processed (10% buffered, formalin-fixed) paraffin-embedded tissue from the files of the Division of Surgical Pathology of the Johns Hopkins Hospital. In brief, after paraffin was removed with xylene and graded alcohols, 6-μm tissue sections were incubated in 3% hydrogen peroxide in methanol for 15 minutes to block endogenous peroxidase activity, and a 1:100 dilution of normal nonimmune serum (goat or horse) was applied

in TRIS–saline for 30 minutes. Then the slides were incubated overnight with affinity-purified polyclonal anti-Hpr or monoclonal anti-Hpr at 2.5 μg/mL in TRIS–saline (pH 7.6) at 4 °C. With intervening washes with TRIS–saline, the sections were incubated successively with biotinylated goat antirabbit or horse antimouse immunoglobulin (1:100; Vector Laboratories) and avidin–horseradish peroxidase complex (1:100; Vector, Burlingame, CA), both for one hour at 25 °C. Aminoethylcarbazole (AEC) (Biomeda Universal Kit, Foster City, CA) was used as a chromogen, with Mayer's hematoxylin counterstaining. As a negative control, TRIS–saline was substituted for the primary antibody in each case; in some cases, diluted (1/20) nonimmune serum from the same species as the secondary antibody was used. A known positive case was used as a positive control in every run. Staining was defined as positive for Hpr if immunoreactivity was present in infiltrating breast carcinoma; granular cytoplasmic immunoreactivity was present without observable nuclear staining; and staining was heterogeneous (i.e., the level of reactivity varied from cell to cell or from region to region in each section). Tumors were graded simply as positive or negative, and each case was examined by at least two authors without their knowledge of the patient's status.

RESULTS

Forty-eight women who died of breast cancer were included in this retrospective autopsy study. The average age at the time of diagnosis was 52 years (standard deviation [SD] = 12; range, 16–78 years). In most cases, the exact stage of disease at diagnosis could not be determined from the available records. In 33 cases, at least one axillary node contained a metastasis; in 11, all identified nodes were free of metastasis, and in 4 cases no information was available on nodal status. Six patients had clinical evidence of systemic metastasis at or around the time of primary tumor diagnosis. The mean disease-free survival length from the time the primary tumor was treated until recurrence or metastatic disease was documented was 3.5 years (SD = 4.2; range, 0–21.4 years). The mean total survival time from diagnosis was 5 years (SD = 5.0; range, four days to 21.4 years).

Immunohistochemical Studies

Thirty-seven of the 48 primary tumors (77%) expressed Hpr epitopes, whereas 11 (23%) were negative (Table 1). Twenty-seven of the positive cases (73%) remained so when either the biopsy specimen of the recurrence or the autopsy material was considered, whereas the status of 10 (27%) was reversed and these cases were negative when the subsequent specimens were examined. In contrast, 10

TABLE 1. CHANGES IN IMMUNOHISTOCHEMICAL
STAINING STATUS WITH ANTI-Hpr IN RECURRENT
TUMORS COMPARED WITH THE STAINING STATUS
OF THE PRIMARY TUMOR IN 48 CASES
OF FATAL BREAST CARCINOMA

Staining Status of Primary Tumor	Cases Retaining Original Staining Status on Recurrence	Cases Reversing Original Staining Status on Recurrence	Total
Negative	1 (9%)	10 (91%)	11 (100%)
Positive	27 (73%)	10 (27%)	37 (100%)
Total	28 (58%)	20 (42%)	48 (100%)

Fisher exact probability $P = 0.0005$.

of the 11 negative primary tumors (91%) were positive on examination of material from the recurrent disease, and only 1 (9%) remained negative. The difference in the proportion of cases in which the staining status (with reference to primary negative and positive staining groups) was reversed was highly significant statistically (Fisher exact $P = 0.0005$).

To further understand temporal changes in Hpr expression and their significance, we evaluated 19 cases separately for which material was available from the primary tumor, the recurrence, and the autopsy specimens, because these cases provided evaluable tissue from three time points. These patients survived for an average of 1.3 years (SD = 2.1; range, 0–12.4 years) after recurrence. Fifteen of the 19 primary tumors (79%) expressed Hpr epitopes. In 12 of the 15 positive cases (80%), both the biopsy specimen of the recurrence and the autopsy material were Hpr positive. In one originally positive case (7%), no staining was observed in the biopsy specimen of the recurrence, but the tumor was positive again at the time of autopsy. Four (21%) of the 19 primary tumors showed no immunoreactivity with anti-Hpr antibodies. All four (100%) of these were Hpr positive at the time of recurrence, and three were also Hpr positive at autopsy.

Among 18 available axillary lymph node metastases that were excised with the primary tumors, 16 were Hpr positive, as were all 4 lymph nodes in cases with Hpr-negative primary tumors.

DISCUSSION

More than 25% of women with early (stage I) breast carcinoma have clinically undetectable metastasis at the time of diagnosis.[5] The recent successes of adjuvant combination chemotherapy for early breast cancer[6–9] and the inability to use traditional histopathologic features to differentiate between clinically aggressive and clinically in-

dolent disease[10] have stimulated a search for molecular markers of prognosis. Frequently, markers are prognostic because they are biologically active molecules that play a direct role in the evolution of a malignant process. For example, epidermal growth factor (EGF) receptor expression in breast cancer is associated with poor prognosis and resistance to antiestrogen therapy[11,12]; *in vitro*, overexpression of the EGF receptor is associated with transformation of NIH-3T3 fibroblasts in the presence of EGF,[13] and monoclonal antibodies against the extracellular domain of the receptor inhibit growth factor-dependent proliferation of malignant cell lines.[14]

Similarly, overexpression of the c-erbB-2/HER-2 oncogene in stage II breast cancer is associated strongly with aggressive disease.[15–19] This clinical observation correlates with observations *in vitro*, because NIH-3T3 fibroblasts that overexpress c-erbB-2/HER-2 are transformed constitutively.[20–22] A mutated rat HER-2 gene placed in transgenic mice results in the development of adenocarcinomas of the breast in all of the male and female animals that express the transform of the gene.[22]

At another level, metalloproteinases such as type IV collagenase may promote tumor invasion by facilitating degradation of basement membranes.[23] Not surprisingly, metalloproteinase expression in clinical tumor tissue correlates with a tendency for metastasis.[24–26] Cathepsin D is a lysosomal enzyme that can degrade basement membranes in acidic environments.[27] High cytosolic concentrations of this protein also are associated with a more aggressive tumor phenotype.[28] Finally, the loss of (or failure to express) a tumor suppressor gene identifies a clinically adverse phenotype and highlights fundamental growth dysfunction. Loss of expression of such suppressor or antioncogenes as RB[29,30] and the recently described Nm 23[31,32] occurs in clinically aggressive neoplasms and may function early in cellular transformation.

Previous studies from this laboratory showed that some breast cancers produce proteins that react with anti-Hpr antibodies and that tumors that do so behave in a clinically

TABLE 2. IMMUNOHISTOCHEMICAL STAINING PATTERN
OF METASTATIC CARCINOMA IN AXILLARY LYMPH NODE
(LN) REMOVED AT THE TIME OF INITIAL DIAGNOSIS AND
COMPARED WITH THE STAINING OF THE PRIMARY
BREAST TUMOR

Primary Tumor Staining	LN Metastasis Negative Staining	LN Metastasis Positive Staining	Total
Negative	0	4	4
Positive	2	12	14
Total	2	16	18

aggressive manner.[1,2] Hpr epitopes were seen in 30% of early (stages I and II) breast cancers, and their expression was associated with a shorter time to tumor recurrence and a significant tendency (4.23 hazard ratio) toward development of recurrent disease.[2]

The data from the current study underscore the link between Hpr-epitope expression and worsened prognosis in primary breast cancer. In this population of breast carcinomas selected for their deadly behavior, 77% of the primary tumors were Hpr positive. This is more than 2.5 times the proportion of positive cases in an unselected group of early breast cancers.[2]

Examined from another standpoint, primary tumors that initially were Hpr negative showed a strong propensity to become Hpr positive at the time of recurrence. In this study, 91% of the primary tumors that initially were negative for Hpr were positive on relapse. In contrast, most (73%) positive primary tumors were Hpr reactive at the time of recurrence, with the staining status of only 27% reversing and becoming negative. Although the change from Hpr positivity to Hpr negativity may represent a true biologic alteration, it also may result from technical factors. All of the specimens that "became" Hpr negative were autopsy samples collected after variable postmortem intervals and processed with variable fixation times; each of these factors might affect the integrity of antigenic determinants. Furthermore, in three of the cases, lymph nodes removed during excision of the primary tumor also were examined and were positive. Technical considerations aside, the difference in the proportion of cases in which the staining status was reversed—with reference to the primary negative- and positive-staining group—was highly significant statistically. This finding suggests that the trend is for negative tumors to become positive with recurrent disease and for positive tumors to remain Hpr reactive. Examination of the subset of 19 cases with both biopsy and autopsy samples supported this conclusion and suggested that, once the tumor acquired Hpr expression, it tended to remain positive for the course of disease.

In conclusion, the expression of Hpr epitopes by breast cancers correlates with aggressive behavior and a poor prognosis. The breast cancer proteins that bear Hpr epitopes may be biologically active, because the increasing incidence of expression goes hand-in-hand with increasing malignancy. Moreover, that expression, once acquired, tends to remain a permanent characteristic of the tumor.

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Original Article

Expression of C-erbB-2 Oncoprotein in Mammary and Extramammary Paget's Disease

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Formalin-fixed, paraffin-embedded tissue sections from 45 patients with mammary and extramammary Paget's disease were stained immunohistochemically with the use of a polyclonal antiserum directed against a 14–amino acid segment of the c-erbB-2 oncoprotein. Positive membrane staining, which correlates with gene amplification, was found in 15 of 19 cases (79%) of mammary Paget's disease, 4 of 13 cases (31%) of vulvar Paget's disease, none of 8 cases of scrotal Paget's disease, and none of 5 cases of perianal Paget's disease. Of the 19 patients with mammary Paget's disease, specimens of underlying breast tissue were available from 14; all contained a concurrent ductal adenocarcinoma. Concordance of c-erbB-2 antigen staining between the underlying breast carcinoma and the pagetoid component was observed in 12 cases. Of the 13 patients with vulvar Paget's disease, 2 had superficial stromal invasion, and 3 had underlying, deeply invasive adenocarcinomas. One superficially invasive case was positive for c-erbB-2 expression. One additional case of vulvar Paget's disease had an associated primary pagetoid endocervical adenocarcinoma that spread into the endometrium; both the endocervical and vulvar components stained positively for the c-erbB-2 antigen. The results of this study indicate that the c-erbB-2 oncoprotein may play a role in the pathogenesis of extramammary Paget's disease. These results also suggest that the c-erbB-2 oncoprotein may function *in vivo* to promote intraepithelial spread of adenocarcinoma cells. (Key words: C-erbB-2; Paget's disease; Breast; Vulva) Am J Clin Pathol 1991;96: 243–247

In humans, the c-erbB-2 oncogene has been found to code for a 185,000-dalton transmembrane glycoprotein with tyrosine kinase–mediated mitogenic activity.[1,2] Amplification of the gene is associated with increased c-erbB-2 oncoprotein expression and has been demonstrated in as many as 30% of adenocarcinomas arising in the breast and ovary, as well as a smaller percentage of adenocarcinomas of the stomach, colon, and salivary glands.[3–7] Several investigators demonstrated correlation of c-erbB-2 amplification and oncoprotein expression with advanced stage of breast carcinoma, early relapse, and shortened overall survival length,[5,8,9] whereas others have not been able to show this association.[10,11] Additional analyses of differential c-erbB-2 oncoprotein expression in invasive

and *in situ* breast carcinomas uniformly have revealed a significantly higher incidence of c-erbB-2 antigen positivity in ductal carcinoma *in situ*, particularly of the comedo type.[12–14] Recently, Lammie and colleagues reported a striking association of c-erbB-2 oncoprotein expression with Paget's disease of the nipple; they found immunohistochemical positivity for c-erbB-2 antigen in 91% of cases tested.[15]

Extramammary Paget's disease is a rare form of adenocarcinoma that has been observed in the vulva, scrotum, perianal skin, axilla, oral mucosa, and eyelid.[16–19] There is still controversy regarding the site and cell type of origin of extramammary Paget's disease.[17] However, recent immunohistochemical evidence suggests that, with the exception of perianal Paget's disease arising from rectal adenocarcinoma, most cases exhibit apocrine differentiation, similar to mammary Paget's disease and breast carcinoma.[20,21] To further assess the relationship between mammary and extramammary Paget's disease, and the possible association between c-erbB-2 oncoprotein expression and pagetoid growth of adenocarcinoma cells, we performed immunohistochemical staining for the c-erbB-2 antigen in 45 cases of mammary and extramammary Paget's disease.

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MATERIALS AND METHODS

Forty-five cases of Paget's disease were obtained from the surgical pathology files of the Vancouver General Hospital, 1975–1990 (33 cases), and the Barnes Hospital, 1983–1989 (12 cases). Sites included the breast in 19 cases, vulva in 13 cases, scrotum in 8 cases, and perianal skin in 5 cases. Tissue blocks from mastectomy specimens were available in 14 of 19 cases of mammary Paget's disease; the remaining 5 cases were only nipple biopsy specimens. Axillary lymph nodes were included with 13 of the breast resection specimens. Vulvectomy specimens were available from all 13 cases of vulvar Paget's disease; 4 included tissues from inguinal lymph node dissections and 1 included a total hysterectomy specimen. All eight cases of scrotal Paget's disease were large resection specimens, including inguinal lymph nodes in one case. Four of the five cases of perianal Paget's disease consisted of wide resection specimens, and one was tissue from a skin biopsy.

Sections from formalin-fixed, paraffin-embedded tissue blocks, including skin and underlying breast or subcutaneous tissue (if available), were cut at 3-μm thickness onto silane-treated slides (2% 3-aminopropyltriethoxysilane in acetone; Sigma Chemicals, St. Louis, MO). After deparaffinization and rehydration, the sections were incubated for 20 minutes in equal volumes of methanol and 3% hydrogen peroxide, washed in 0.5 mol/L TRIS buffer, and blocked for 10 minutes with nonimmune goat serum. Primary polyclonal antiserum directed against a synthetic 14-amino acid polypeptide, representing the carboxy-terminus of the c-erbB-2 protein (the gift of Dr. Yosh Teramoto, Triton Biosciences Inc., Alameda, CA), was applied to each slide at a 1:1,000 dilution for overnight incubation.

After 0.05 mol/L TRIS buffer washes, each section was incubated for 60 minutes with a 1:200 dilution of peroxidase-conjugated goat–antirabbit antibody (Jackson Immunoresearch, West Grove, PA), washed in 0.1 mol/L TRIS–acetate buffer (pH 5.0), and developed for 10–12 minutes in chromogen (6 mg amnioethylcarbazole [AEC] and 1.5 mL dimethylformamide [both from Sigma], in 287 mL 0.1 mol/L TRIS–acetate buffer with 0.5 mL 3% hydrogen peroxide). Sections then were washed in water, counterstained with hematoxylin, and mounted in aqueous medium. Positive controls consisted of two cases of breast carcinoma with immunohistochemical c-erbB-2 antigen positivity, confirmed by Western blot analysis (personal communication, Dr. Yosh Teramoto). Negative controls consisted of Paget's disease cases incubated with nonimmune rabbit serum or absorbed primary antiserum. Diluted antiserum was incubated for 18 hours at 4 °C with an equal volume of the 14–amino acid immunogenic c-erbB-2 peptide sequence (Triton Biosci-

ences) at 50 μg/mL. Sections were assessed for the presence of cell membrane staining only, which has been shown to correlate most closely with gene amplification.[11] All membrane staining was abolished by antiserum absorption.

RESULTS

Histologic examination of cases of mammary and extramammary Paget's disease revealed variably hyperplastic epidermis containing adenocarcinoma cells distributed singly and in groups or acini throughout the epidermis. All 14 mastectomy specimens also contained an underlying ductal carcinoma, with 7 having an infiltrative component. *In situ* comedocarcinoma was identified in four patients. Dermal lymphatic involvement was not seen in any of the breast specimens. All 13 axillary lymph node dissection specimens were negative for metastatic disease. Of the 13 vulvar Paget's disease cases, 8 showed involvement of the epidermis and follicular infundibula only. Two showed superficial dermal invasion, three contained deeply invasive adenocarcinomas (with regional lymph node metastases in two), and one case without vulvar dermal invasion was associated with a poorly differentiated microinvasive cervical adenocarcinoma with a pagetoid growth pattern in the cervix and the endometrium, but without clinical evidence of vaginal disease. Two of eight cases of scrotal Paget's disease were associated with deeply invasive adenocarcinoma, and two showed superficial invasion; tissue from a single lymph node dissection was negative for metastases. Two of five cases of perianal Paget's disease were associated with mixed signet-ring cell/colloid carcinomas of distal rectal origin.

Immunohistochemical analysis for c-erbB-2 antigen revealed positive membrane staining in 15 of 19 cases (79%) of mammary Paget's disease and 4 of 13 cases (31%) of vulvar Paget's disease. All examples of scrotal and perianal Paget's disease were negative for membrane staining. The c-erbB-2-positive adenocarcinoma cells contrasted clearly with the surrounding negative keratinocytes. Several cases with membrane staining also exhibited granular cytoplasmic staining. The pattern of pagetoid growth did not differ between c-erbB-2–positive and –negative cases.

Of the 14 cases of mammary Paget's disease for which mastectomy specimens containing underlying ductal carcinoma were available for analysis, 12 were positive for c-erbB-2 staining (Fig. 1). Concordant c-erbB-2 staining was observed in 12 of these 14 cases; 2 with c-erbB-2 positivity of the Paget's disease component were negative in the underlying breast carcinoma. However, both of these cases had only single, microscopic foci of ductal carcinoma *in situ;* hence, regional variation of c-erbB-2 expression and sampling error could account for this dis-
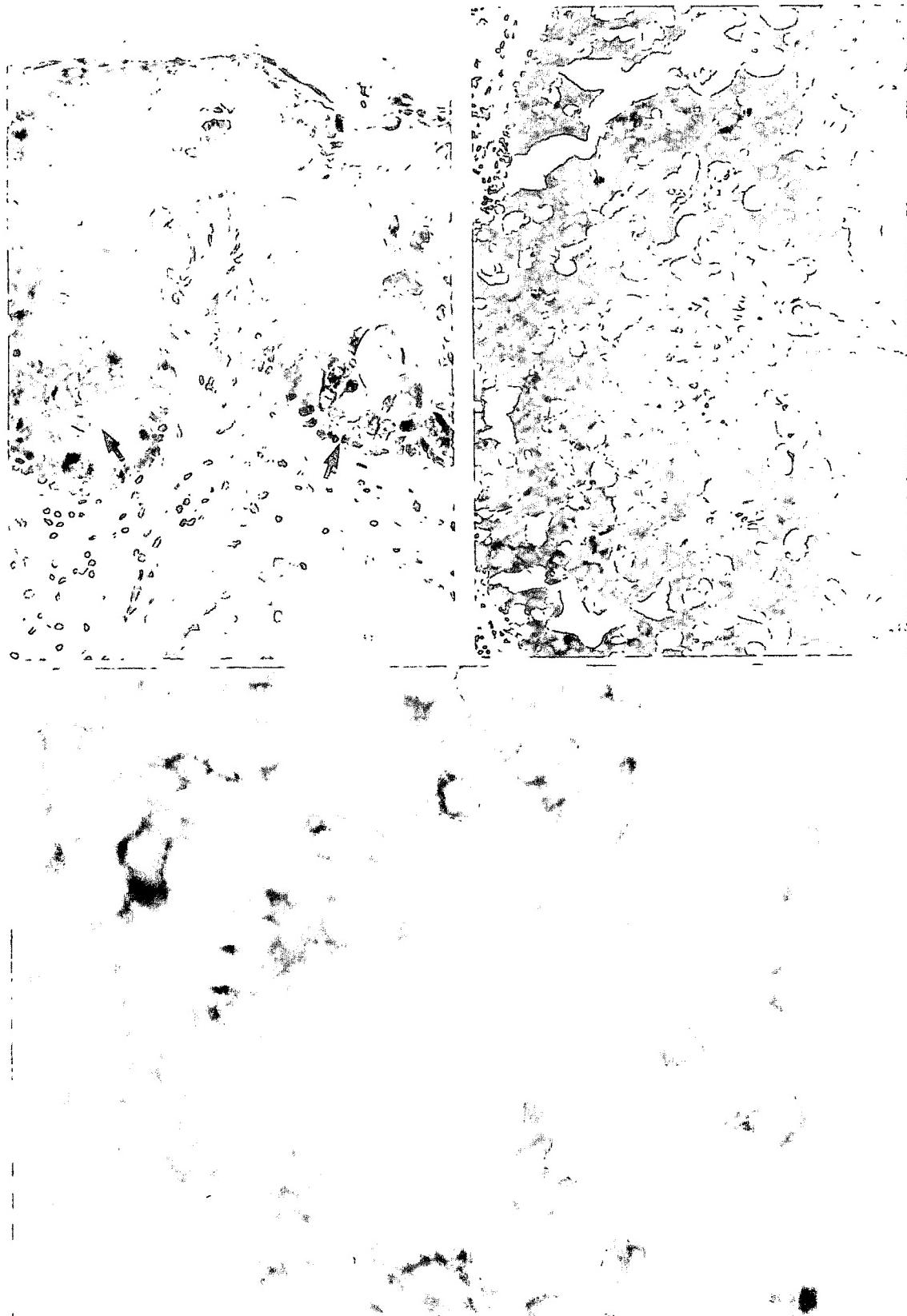
FIG. 1 (*upper, left*). Mammary Paget's disease, showing c-erbB-2 membrane staining of basally located (*arrows*) adenocarcinoma cells. Amnioeth·l-carbazole with hematoxylin (×400).

FIG. 2 (*upper, right*). *In situ* ductal carcinoma of breast, comedo type, showing c-erbB-2 membrane and cytoplasmic staining. Amnioethylcarbazcle with hematoxylin (×400).

FIG. 3 (*lower*). Vulvar Paget's disease, positive for c-erbB-2 membrane staining. Amnioethylcarbazole with hematoxylin, dark blue filter (×800).

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crepancy. The four cases containing an *in situ* comedo component were strongly positive for c-erbB-2 membrane staining (Fig. 2). Six of seven cases with an infiltrative ductal carcinoma component were c-erbB-2 positive.

Of the four cases of c-erbB-2–positive vulvar Paget's disease (Fig. 3), two contained intraepidermal adenocarcinoma only, one showed superficial stromal invasion, and one was associated with a poorly differentiated endocervical adenocarcinoma. The endocervical carcinoma also exhibited strong c-erbB-2 membrane staining (Fig. 4). To our knowledge, this is the first reported example of immunohistochemical c-erbB-2 antigen expression in an endocervical adenocarcinoma. In five additional cases of typical endocervical adenocarcinoma, subsequent analysis for c-erbB-2 antigen expression produced negative findings in all cases. Both cases of vulvar Paget's disease with lymph node metastases were negative for c-erbB-2 staining.

Because of the small number of cases in this series, patient follow-up data were not analyzed statistically.
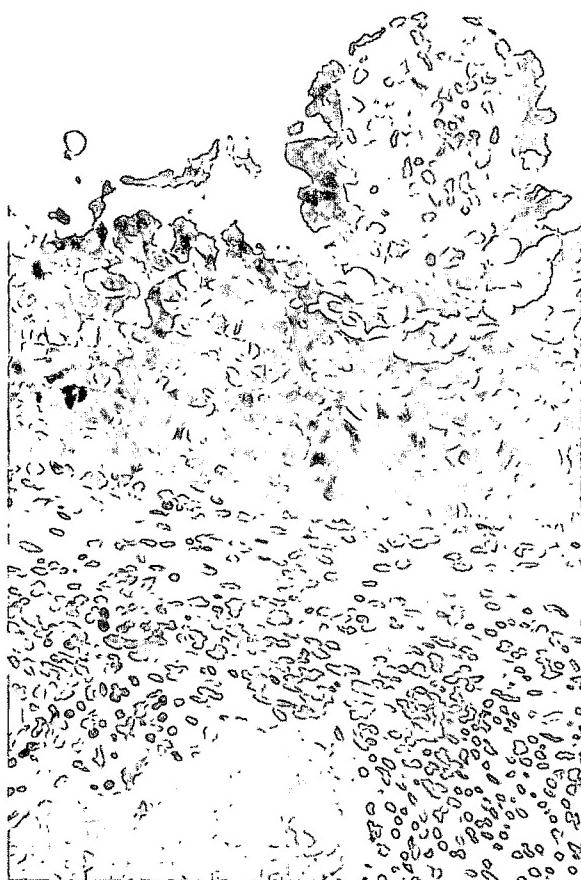
FIG. 4. Endocervical adenocarcinoma with pagetoid growth pattern from the same patient as the specimen shown in Figure 3, with c-erbB-2 membrane staining. Amnioethylcarbazole with hematoxylin (×400).

Nevertheless, none of the four patients with c-erbB-2–positive vulvar Paget's disease has had lymph node metastases develop, although two have had local tumor recurrence.

DISCUSSION

Although the incidence of amplification and increased expression of the c-erbB-2 oncogene has been estimated at 30% in breast carcinomas,[5] recent evidence has indicated that this figure varies widely between purely *in situ* and invasive tumors. Barnes and co-workers[11] found immunohistochemical staining for c-erbB-2 antigen in only 9% of purely invasive adenocarcinomas, whereas Gusterson and associates and Van de Vijver and colleagues found positive staining in 44% and 42% of cases of pure *in situ* ductal carcinoma, respectively.[13,14] Similarly, Allred and associates found c-erbB-2 oncoprotein expression in 11% of infiltrative ductal carcinomas and 55% of *in situ* ductal carcinomas.[12] These results seem to be at odds with those of other investigators, who have found an association between c-erbB-2 positivity, advanced stage of disease, and shortened survival time.[5,8,9] This apparent paradox may be explained in part by the additional observation that 80–100% of *in situ* ductal carcinomas with high-grade comedo morphologic characteristics are positive for the c-erbB-2 antigen.[12,14] The presence of a comedocarcinoma pattern has been associated with early local recurrence in patients with breast carcinomas treated by lumpectomy,[22] possibly indicating extensive intraductal tumor spread.

The current study documents c-erbB-2 protein expression in 79% of cases of mammary Paget's disease, a proportion similar to that found by Lammie and associates.[15] We also have identified c-erbB-2 positivity in 31% of cases of vulvar Paget's disease, a previously unrecognized association. A recent study by Meissner and colleagues[23] showed similar expression of c-erbB-2 oncoprotein in mammary Paget's disease but did not demonstrate it in seven cases of vulvar Paget's disease. Because c-erbB-2 expression was absent in their relatively small series of vulvar cases, the authors concluded that mammary and extramammary Paget's disease, although phenotypically similar, derive from different genetic alterations. Conversely, the findings of our study suggest that c-erbB-2 oncoprotein expression may play a role in the pathogenesis of extramammary Paget's disease and would tend to support immunohistochemical studies indicating a common derivation for some forms of breast carcinoma and vulvar Paget's disease.[20,21] However, c-erbB-2 expression clearly is not specific to carcinomas of apocrine origin, because it has been identified in adenocarcinomas of the ovary and gastrointestinal tract[5-7] and was present in our unusual case of vulvar Paget's disease with an associated

cervical adenocarcinoma. More importantly, the combined observations of increased c-erbB-2 oncoprotein expression in a high proportion of cases of Paget's disease and of intraductal comedocarcinoma of the breast, as well as a significant proportion of vulvar Paget's disease cases, suggest that this oncoprotein may function *in vivo* to promote intraepithelial tumor cell proliferation. The apparent biochemical function of the transmembrane c-erbB-2 protein as a tyrosine kinase-mediated activator of cell proliferation conforms with this hypothesis.[1,2]

The results of this study suggest areas that may need to be investigated regarding the role of c-erbB-2 oncoprotein in intraepithelial tumor growth. Retrospective and prospective studies of c-erbB-2 expression in patients with breast cancer treated by lumpectomy may indicate which patients are at greatest risk for subsequent local tumor recurrence. Additional larger studies of patients with vulvar Paget's disease—the prognosis of which currently is predicted by the presence of stromal invasion and lymph node metastasis[16]—also may provide information regarding the likelihood of local tumor recurrence.

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Cell-Surface Ganglioside GD2 in the Immunohistochemical Detection and Differential Diagnosis of Neuroblastoma

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The expression of the disialoganglioside GD2 was analyzed in 67 solid tumors and normal tissues from children by using the GD2-specific murine monoclonal antibody 3A7 and the indirect immunoperoxidase method. GD2 was expressed in all of 28 neuroblastomas and was most abundant in stroma-poor tumors. In differentiating stroma-rich neuroblastomas, neuroblastic clusters, neurofibrils, and most ganglion-like cells were positive, whereas Schwann's-cell stroma did not express GD2. In ganglioneuromas, only a few ganglion-like cells showed GD2, whereas all other structures were negative. Scattered foci of ganglioside GD2 also were found in some non-neuronal tumors, such as rhabdomyosarcomas and osteosarcomas, but not in lymphomas, Askin tumors, or most Wilms' tumors. The monoclonal antibody 3A7 is a useful aid in the immunohistochemical diagnosis of neuroblastoma. In addition, the intense cell surface staining of neuroblastoma cells by this reagent makes it potentially useful for detecting residual neuroblastoma in bone marrow samples and lymph node biopsies. (Key words: Pediatric neoplasms; Soft tissue neoplasms; Neuroectodermal tumors; Sarcomas) Am J Clin Pathol 1991;96:248–252

Most neuroblastomas are "small round-cell tumors," with a variety of possible differential diagnoses, including non-Hodgkin's lymphomas, Askin's tumors, Ewing's sarcomas, other small-cell sarcomas, and primitive neuroectodermal tumors.[1,2] Therefore, the specific histopathologic diagnosis of neuroblastoma is aided by several immunohistochemical markers, such as neurofilaments,[3,4] synaptophysin,[5-8] neural cell adhesion molecule,[9] and neuron-specific enolase.[10-13] However, none of these markers is specific to neuroblastoma, and the antibodies against these proteins label a variety of other tumors of childhood.[2,14] Furthermore, in immunohistochemical analysis, they are not helpful in assessing prognosis.[15] Other, as yet uncharacterized, monoclonal antibodies have proved to be promising in differentiating neuroblastoma from other primitive peripheral neuroectodermal tumors.[16,17]

The disialoganglioside GD2 is a cell surface glycolipid that is synthesized in abundance by primary untreated neuroblastomas[18-21] and can be detected in the plasma of patients with these tumors.[20-22] Circulating GD2 decreases in response to therapy and reappears in patients whose disease has recurred.[23] Thus, GD2 has been introduced as a potential marker for neuroblastoma.[18,19,21] However, by chromatographic techniques, GD2 has not been found in well-differentiated ganglioneuroblastomas.[19] Other neural crest-derived tumors, such as neuroepithelioma and pheochromocytoma, do not synthesize GD2, whereas this marker is a major ganglioside in melanomas and gliomas.[24-26]

In the current study, the differential diagnostic value of the anti-GD2 monoclonal antibody 3A7[10] was evaluated by indirect immunoperoxidase staining of malignant and benign solid neoplasms of childhood. Strong staining by 3A7 was seen in all 28 neuroblastomas analyzed, whereas most other childhood neoplasms, including Wilms' tumors, lymphomas, and other neuroectodermal tumors, showed little if any reactivity.

MATERIALS AND METHODS

Materials

Case materials consisted of fresh frozen tissues from pediatric solid tumors, obtained either by surgery or biopsy

at initial presentation or after chemotherapy. The samples were frozen in liquid nitrogen and cryopreserved at −70 °C until they were analyzed. Twenty-eight neuroblastomas (including five well-differentiated stroma-rich ganglioneuroblastomas) were analyzed. The diagnosis was based on light and electron microscopy of the tumors, immunohistochemical demonstration of neurofilaments and synaptophysin, and elevated urinary cathecolamine secretion. Other solid tumors of childhood included lymphoma (N = 6), Wilms' tumor (7), renal malignant rhabdoid tumor (1), rhabdomyosarcoma (10), ganglioneuroma (3), alveolar soft part sarcoma (1), small-cell desmoplastic tumor of childhood (1), Askin's tumor (2), osteogenic sarcoma (5), synovial sarcoma (1), and anaplastic sarcoma of the urinary tract (1). Biopsies of normal brain, sympathetic ganglion, adrenal gland, striated muscle, lymph node, thyroid gland, gut, myocardium, spleen, thymus, liver, pancreas, and kidney were obtained from autopsies of neonates (2) and fetuses of 20 to 22 weeks' gestation (2).

All samples were frozen in liquid nitrogen, cut at 10 μm with a cryostat (Cambridge Instruments GmbH, Nussloch, FRG), air-dried at room temperature for 30 minutes, fixed for 10 minutes in cold (−20 °C) acetone, and stained by the indirect immunoperoxidase method, as described here.

Immunohistochemistry

The murine monoclonal antibody (MoAb) 3A7 is of the IgM class and specifically labels the cell-surface disialoganglioside GD2.[18] 3A7 is not commercially available and was provided by Dr. N-K.V. Cheung, the Memorial Sloan Kettering Cancer Center, New York, New York. MoAb 3A7 was used as a hybridoma supernatant. It was diluted at 1:6 in Tris-buffered saline (TBS) containing 0.1% bovine serum albumin (pH 7.4). The final concentration of the antibody was 3 μg/mL. Sections were incubated for 10 minutes in 10% normal rabbit serum, blotted, incubated with the primary antibody for 30 minutes, and washed three times for 5 minutes in TBS. The secondary antibody, a horseradish peroxidase-labeled rabbit antimouse immunoglobulin (Dakopatts P-161, Copenhagen, Denmark), was diluted at 1:400 and incubated for 30 minutes at room temperature. After three further washes in TBS, sections were immersed for 20 minutes at room temperature in a freshly made solution of 3-amino-9-ethylcarbazole (40 mg) in 12 mL of N, N-dimethylformamid mixed in 200 mL of acetate buffer (0.05 M, pH 5.0) and 200 μL of hydrogen peroxide. The sections were counterstained with hematoxylin and mounted in Aquamount® (BDH Limited, Poole, UK).

In the preparation of negative controls, the primary antibody was omitted. In addition, before the 3A7 antibody incubation, some sections were fixed for 5 minutes

in methanol. This solvent effectively dissolves the GD2 ganglioside. Only frozen sections of tumor or tissue were used in the study. The 3A7 antibody is not applicable to paraffin-embedded samples because gangliosides are dissolved during tissue processing.

RESULTS

The use of MoAb 3A7 in immunohistochemical studies demonstrated that all 28 neuroblastomas expressed the GD2 ganglioside (Fig. 1A). 3A7 reacted strongly with the membranes of the neuroblastoma cells. Although all neuroblastomas were labeled with 3A7, staining intensity was dependent on the differentiation of the tumor cells. The strongest staining reaction was obtained in stroma-poor undifferentiated neuroblastomas, in which all tumor cells evenly expressed GD2. In differentiating neuroblastomas, neurofibrils were regularly stained, whereas most mature ganglion-like cells in ganglioneuroblastomas and the Schwann's-cell stroma in stroma-rich neuroblastomas were negative for GD2. Little if any GD2 was seen in ganglioneuromas, in which only a few ganglion-like cells bound 3A7 (Fig. 1B). The reactivity of neuroblastoma cells did not decrease during cryopreservation (up to 72 months). Methanol fixation for 5 minutes abolished the staining reaction.

Other "small, round, blue-cell tumors" of childhood, such as lymphoma and Askin's tumor, did not express GD2. Scattered small foci of positive cells were found in rhabdomyosarcomas and Wilms' tumors, in which occasional positive areas were found in the stroma. GD2-positive cells were not seen in blastemal or tubular areas of Wilms' tumors. The undifferentiated cellular areas of most osteogenic sarcomas were labeled by 3A7 (Fig. 2). These results are summarized in Figure 3.

In normal neonatal and embryonic tissues (20–22 weeks' gestation), binding of 3A7 was found in the capsule of the parathyroid gland and the thymus; neuroendocrine cells of the lung; and the peripheral nerves. The adrenal gland, brain, liver, kidney, pancreas, striated and smooth muscle, gut, myocardium, aorta, and sympathetic ganglia were negative.

DISCUSSION

The monoclonal antibody 3A7 appears to be a sensitive marker for neuroblastoma and is a good adjunct in the differential diagnosis of "small round-cell tumors" of childhood. All 28 neuroblastomas in this series were positive by immunoperoxidase staining with this antibody.

The ganglioside GD2 was abundant in undifferentiated neuroblastic areas, but it was not expressed in well-differentiated, stroma-rich areas of neuroblastomas. Ganglioneuromas generally were negative for GD2; only a

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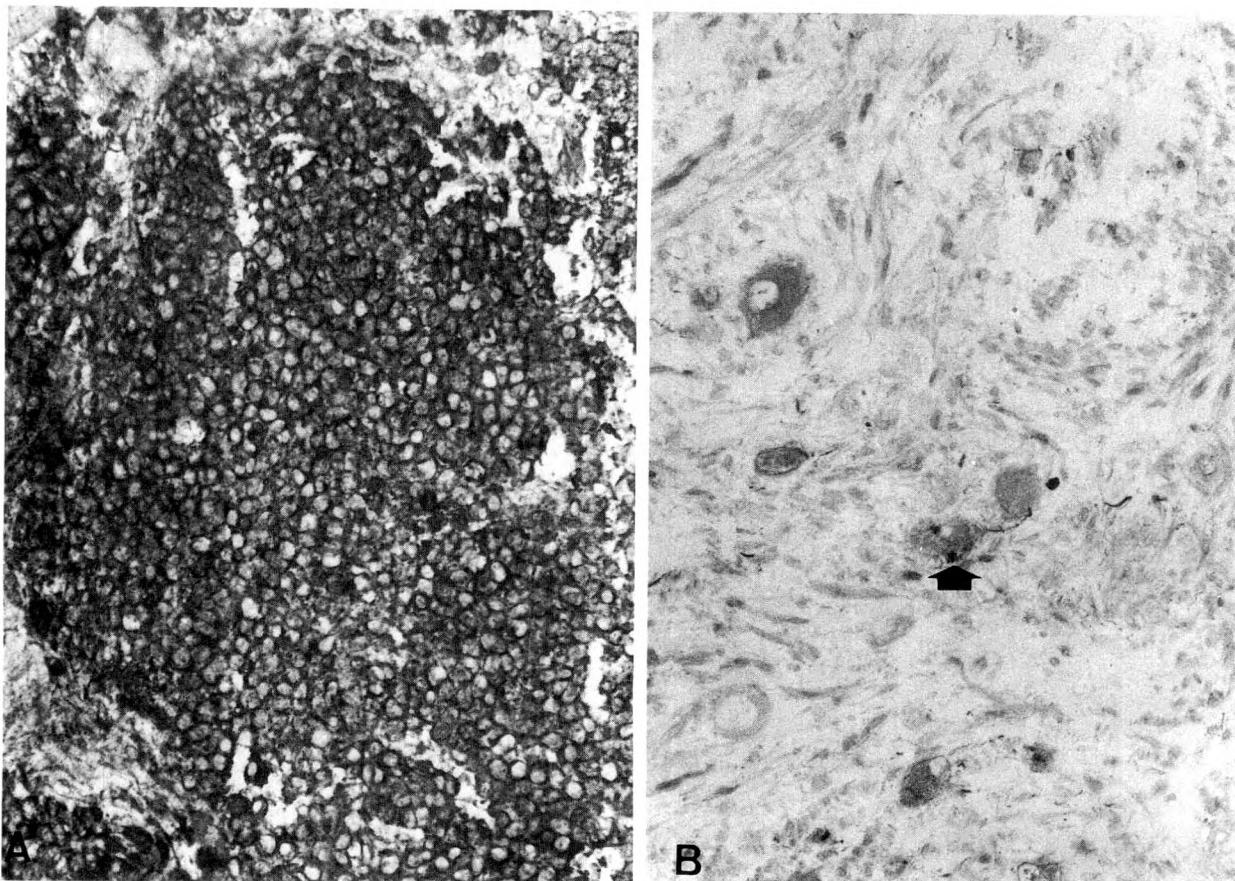
FIG. 1. Binding of the GD2-specific antibody 3A7 to (*A*) stroma-poor, undifferentiated neuroblastoma and (*B*) ganglioneuroma. Note that a ganglion-like cell marked by an arrow shows expression of GD2. *A* and *B*, indirect immunoperoxidase staining of frozen sections (×250).
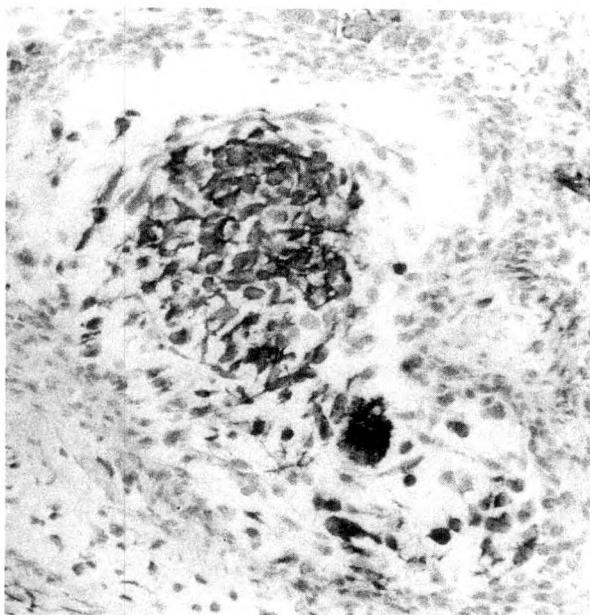

FIG. 2. Binding of the 3A7 antibody to osteogenic sarcoma. Primitive cellular areas are positive, whereas more mature foci do not bind the antibody. Indirect immunoperoxidase staining of frozen sections (×250).

few ganglion-like cells were labeled by 3A7. This finding is analogous to the normal developmental shift in the brain from synthesis of mono- and disialated gangliosides to the more complex multisialated gangliosides.[27,28] Our results suggest that the immunoperoxidase technique with MoAb 3A7 may be more sensitive than thin-layer chromatography[19] in the detection of ganglioside GD2 in tumor samples.

The ganglioneuromas we studied generally were negative for GD2. In neuroblastomas, neurofibrils were labeled by 3A7. Therefore, the loss of ganglioside GD2 may not be explained solely through neuronal differentiation of the tumor cells but may reflect the amount of Schwann's-cell stroma in such tumors. The antibody 3A7 also may be a useful aid in assessing the tumors according to the classification of Chatten and colleagues,[29] which is based in part on the level of stromal differentiation.

Some other pediatric solid tumors, such as osteogenic sarcomas, some rhabdomyosarcomas, and one of seven Wilms' tumors, showed scattered foci of GD2-reactivity. This is in accord with the findings of Cheung and colleagues,[18] who noted reactivity with 3A7 in three of six

Anti-GD2 Monoclonal Antibody in Neuroblastoma Detection
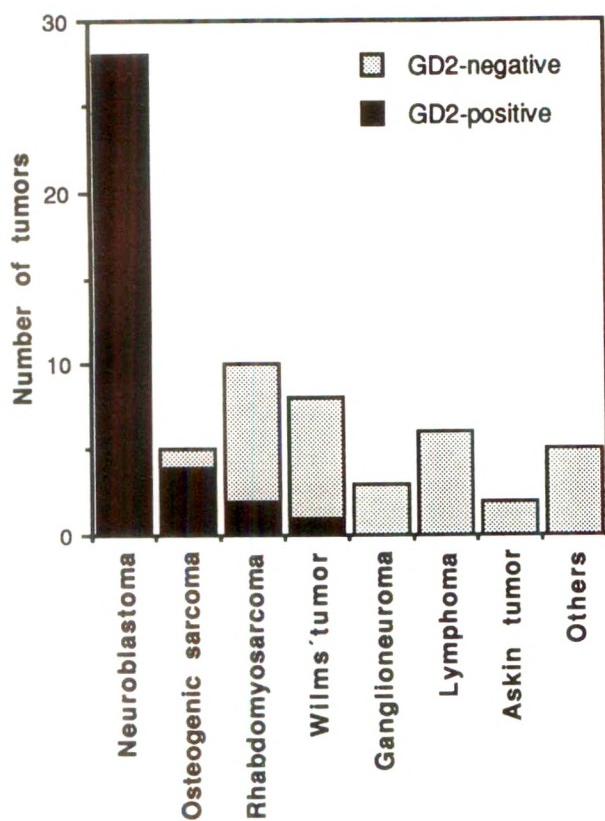
FIG. 3. Binding of the GD2-specific antibody 3A7 to pediatric solid tumors. (Others = synovial sarcoma, small cell desmoplastic tumor of childhood, renal malignant rhabdoid tumor, alveolar soft part sarcoma, and anaplastic sarcoma of the urinary tract.)

osteogenic sarcomas and one of six rhabdomyosarcomas by using the ELISA method. In addition, several Ewing's sarcoma cell lines express GD2,[30] but we do not know whether this is the case with *in vivo* human tumors. Heterogeneous expression of neuronal cell lineage markers is not a rare feature among pediatric tumors, and it has been observed earlier with all neuroblastoma marker proteins.[2] This also is true for primitive neuroectodermal tumors of the central nervous system, which may express all classes of intermediate filaments.[31] Therefore, several marker proteins must be analyzed simultaneously in the proper immunohistochemical evaluation of neuroblastoma and other neuroepithelial tumors.

Whereas the ganglioside GD2 was found in some neural crest derivatives of neonatal and embryonic tissues, we did not observe it in fetal adrenal medulla, which often contains clusters of neuroblastic cells. These have been called "neuroblastomas *in situ*" by some authors.[32] Although adrenal neuroblastic clusters mimic neuroblastoma cells morphologically, our findings are consistent with their biologic behavior, showing that these clusters do not normally progress to neuroblastoma.[33]

We conclude that immunohistochemical studies with the anti-GD2 monoclonal antibody 3A7 are helpful in the detection and differential diagnosis of neuroblastoma. In addition, antibodies against GD2 have proven to be useful therapeutically in antibody-mediated binding of radioactive molecules to neuroblastoma cells[34] and in detecting residual neuroblastoma in bone marrow aspirates.[35] The role of the ganglioside GD2 as a differentiation-related antigen during neuronal maturation merits emphasis and further study.

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Pseudosarcomatous Fibromyxoid Tumor of the Prostate

A Case Report with Immunohistochemical, Electron Microscopic, and DNA Flow Cytometric Analysis

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AND ALBERTO G. AYALA, M.D.[2]

A 22-year-old man presented with symptoms of urinary obstruction and was found to have a 5-cm mass protruding from the left side of the prostate into the prostatic urethra. The lesion was partially removed by transurethral resection (TURP). The patient's symptoms recurred, so he required another TURP four months later; at latest follow-up (four years later), there was no evidence of disease. Light microscopy revealed a myxoid lesion characterized by an atypical fibroblastic proliferation associated with a prominent inflammatory component and granulation tissue-type vasculature. The lesion nearly replaced the prostatic parenchyma and invaded the bladder wall. Immunohistochemistry and electron microscopy showed a predominance of fibroblasts with occasional myofibroblasts. DNA flow cytometric analysis showed that the tumor cells had a diploid DNA content. Given these findings and the indolent clinical course, the authors think that this lesion represents a benign, reactive process consistent with a pseudosarcomatous fibromyxoid tumor, a recently described rare lesion of the genitourinary tract. (Key words: Pseudosarcoma; Pseudotumor; Prostate; Fibromyxoid tumor) Am J Clin Pathol 1991;96:253–258

Benign fibroblastic tumor-like lesions of the visceral organs represent a large and diverse group of pathologic entities that may cause diagnostic difficulties because of their histologic similarities to malignant neoplasms.[1,2] Such pseudomalignant lesions in the genitourinary tract are rare, and have been the subject of case reports during the past decade.[3-11] Those that occur after surgical trauma (for example, after instrumentation, biopsy, transurethral resection, or hysterectomy) have been named "postoperative spindle cell nodules" by Proppe and associates[7] and are thought to represent reactive processes. Others occur in patients without a recent history of surgical trauma,[3,5,6,8-11] and they have been designated variously as "pseudosarcomas," "inflammatory pseudotumors," and, as in an earlier report from our institution of two

cases in the urinary bladder,[8] "pseudosarcomatous fibromyxoid tumors" (PFMTs). Here we report an additional case of PFMT that arose in the prostate, and we review the relevant literature on this lesion.

REPORT OF A CASE

A 22-year-old man first noticed urinary urgency and a gradual decrease in the force of his urinary stream in March 1986. In June 1986, he developed near-total urinary obstruction and was seen by a local physician. A diagnosis of prostatitis was made, and treatment with antibiotics resulted in modest clinical improvement. Urinary obstructive symptoms continued, and cystoscopy in October 1986 revealed an asymmetrical enlargement of the prostate. On the left side, the prostate protruded across the verumontanum, filling two-thirds of the prostatic urethra. The urethral mucosa was intact, and the bladder was unremarkable. Transrectal biopsy of the left prostatic mass was performed, and a diagnosis of "possible low-grade sarcoma" was rendered. The patient was referred to M. D. Anderson Cancer Center for further evaluation and treatment.

Physical examination of the patient at our hospital was unremarkable except for an enlarged and firm prostate. Results of routine hematology and chemistry surveys were within normal limits. Radiologic studies, including chest roentgenography and abdominal and pelvic computed tomography, were negative for metastatic disease. Because of worsening symptoms of urinary obstruction, the patient underwent transurethral resection of the prostate (TURP) in July 1987. The lesion was too large to remove by limited resection; four months later, the patient again de-

veloped symptoms of urinary retention and underwent another TURP to excise the remaining mass. At last contact, in June 1990, he was well with no evidence of genitourinary disease.

MATERIALS AND METHODS

Tissue samples obtained from the two TURP procedures were fixed in 10% phosphate-buffered formaldehyde and embedded in paraffin. Sections measuring 4–5 μm were stained with the hematoxylin and eosin, alcian blue at pH 2.7, periodic acid-Schiff (PAS), and mucicarmine techniques.

Immunoperoxidase staining was performed on representative sections of formalin-fixed, paraffin-embedded tissue by using the avidin-biotin-peroxidase complex method of Hsu and co-workers.[12] The sections were labeled with antibodies against vimentin (DAKO Corporation, Santa Barbara, CA; 1:25 dilution), cytokeratin (AE1/AE3, Boehringer Mannheim, Indianapolis, IN; 1:300), desmin (DAKO; 1:100), alpha-smooth-muscle actin (Sigma Chemicals, St. Louis, MO; 1:1,000), myoglobin (DAKO; 1:1,200), and S-100 protein (DAKO; 1:1,000).

Immunohistologic reactions were visualized using 3-amino-9-ethylcarbazole as chromogen. The slides were counterstained with Mayer's hematoxylin.

For electron microscopic evaluation, fresh tissue samples were fixed in 2% glutaraldehyde, postfixed in 1% osmium tetroxide, and stained with uranyl acetate and lead citrate. Ultrathin, Epon®-embedded sections (Epon; Ted Pella Inc., Redding, CA) were examined.

DNA flow cytometric analysis was performed by a previously described two-step staining method, using acridine orange.[13] Cellular RNA/DNA content was analyzed on a Coulter Profile® (Hialeah, FL) cytophotometer.

RESULTS

Both of the TURP specimens consisted of multiple irregular fragments of soft tissue, ranging in color from tan to pink, measuring about 5.0 × 5.0 × 3.0 cm and 3.0 × 3.0 × 3.0 cm, respectively. Light microscopic evaluation of the lesion revealed a cellular, myxoid proliferation (Fig. 1) replacing the prostatic ducts and acini and extending
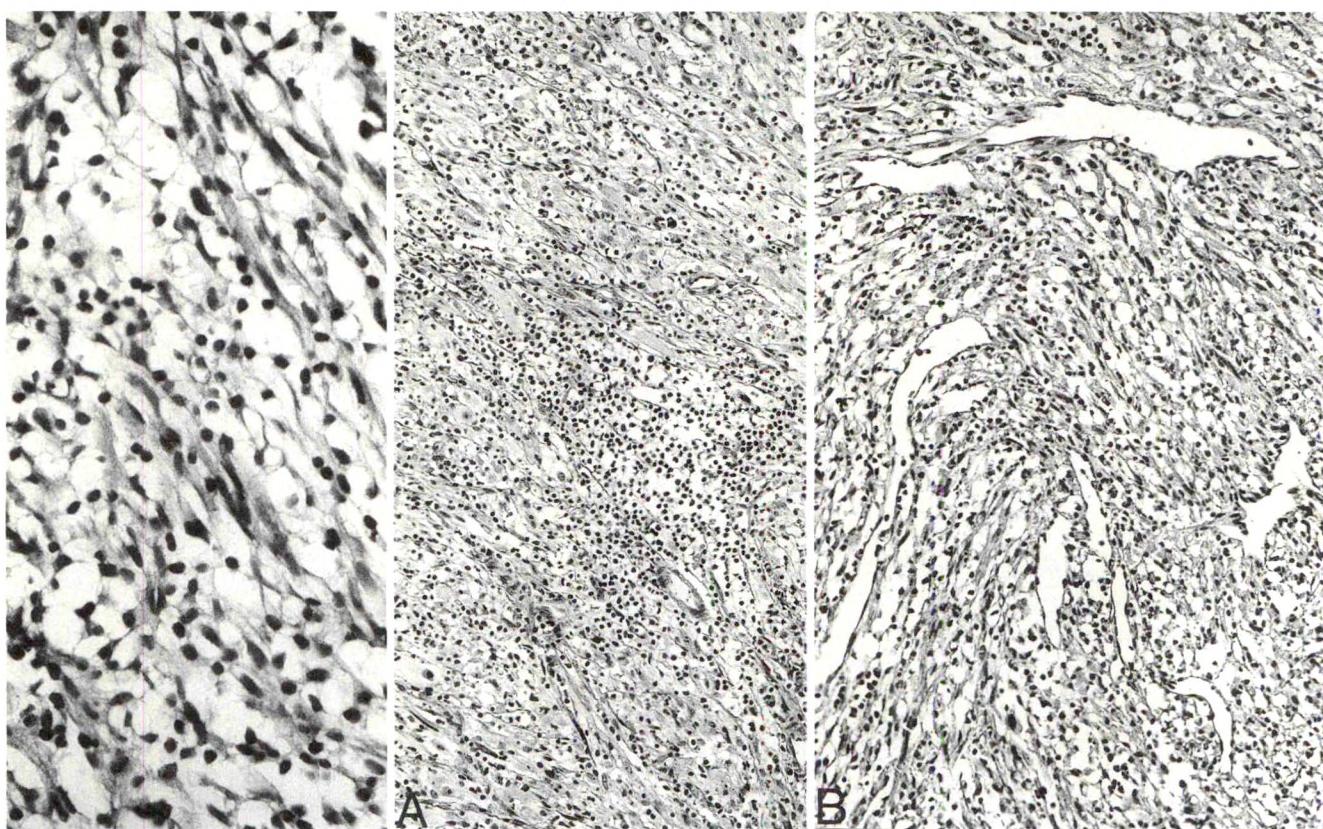
FIG. 1 (*Left*). Tissue obtained by the first transurethral resection shows almost complete replacement of the prostatic parenchyma by a proliferation of spindle-shaped cells lying in a myxoid stroma. Hematoxylin and eosin (×250).

FIG. 2. *A.* The stroma has a loose, edematous appearance and contains chronic inflammatory cells and small vessels. Hematoxylin and eosin (×100). *B.* Higher magnification shows slit-like vessels. Hematoxylin and eosin (×160).

into the bladder wall. The lesion was composed of widely separated spindle cells lying in a loose, edematous, and myxoid stroma that contained chronic inflammatory cells and abundant small vessels (Fig. 2). The nuclei of the spindle cells were large and moderately hyperchromatic and pleomorphic. There were occasional atypical, large cells with bizarre nuclei and prominent single or multiple nucleoli (Fig. 3). Although a few mitoses (average 1 per 10 high-power fields) were present (Fig.4), none were atypical. The stroma was rich in acid mucopolysaccharides, as evidenced by intense alcian blue staining and negativity with the PAS and mucicarmine stains. The spindle cells did not stain for mucin.

The fusiform cells showed strong, intracytoplasmic positivity for vimentin. Scattered cells also were positive for smooth-muscle actin, but no reactivity for desmin, myoglobin, S-100 protein, or cytokeratin was observed.

Electron microscopic analysis revealed elongated cells embedded in a loose intercellular matrix. The cells had oval nuclei, which sometimes were indented. The cytoplasm contained abundant rough endoplasmic reticulum, and there were prominent cell processes (Fig. 5). Some of the spindle cells showed peripheral condensations of microfilaments that occasionally were associated with dense bodies, indicating myofibroblastic differentiation (Fig. 6).

Flow cytometric analysis, performed on fresh tissue from both TURPs, revealed that the cells had a diploid DNA content. The $S+G_2M$ value (proliferative index) was 8% in tissue from the first TURP and 6% in the second specimen.

DISCUSSION

A variety of benign, reactive processes in the genitourinary tract can assume a spindle cell appearance and be confused with malignant mesenchymal neoplasms.[1,2] Reactive pseudosarcomatous spindle cell proliferations related to previous surgery (postoperative spindle cell
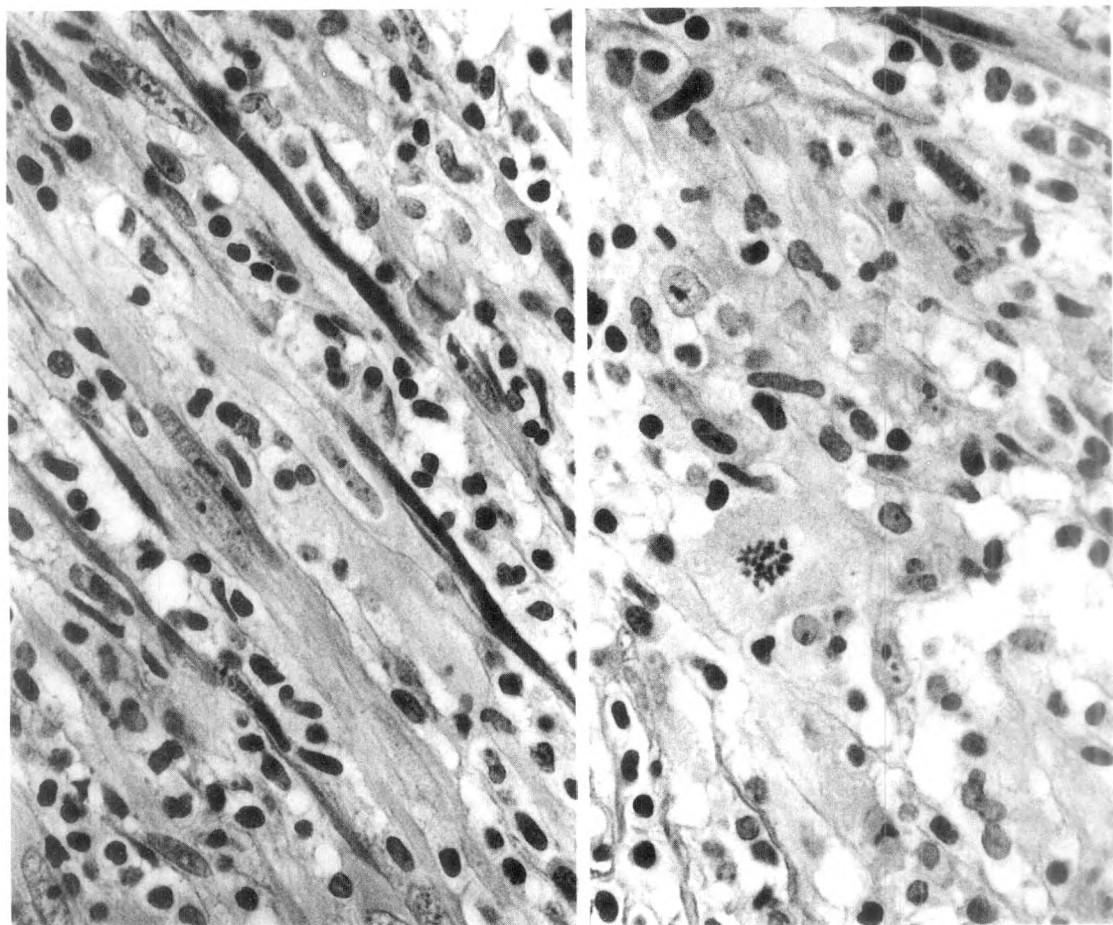
FIG. 3 (*left*). Although the majority of the spindle-shaped cells have uniform, normochromic nuclei with a fine chromatin pattern, there are scattered atypical cells showing large nuclei with prominent nucleoli. Hematoxylin and eosin (×400).

FIG. 4 (*right*). Occasional mitotic figures are present, none of them atypical. Hematoxylin and eosin (×400).
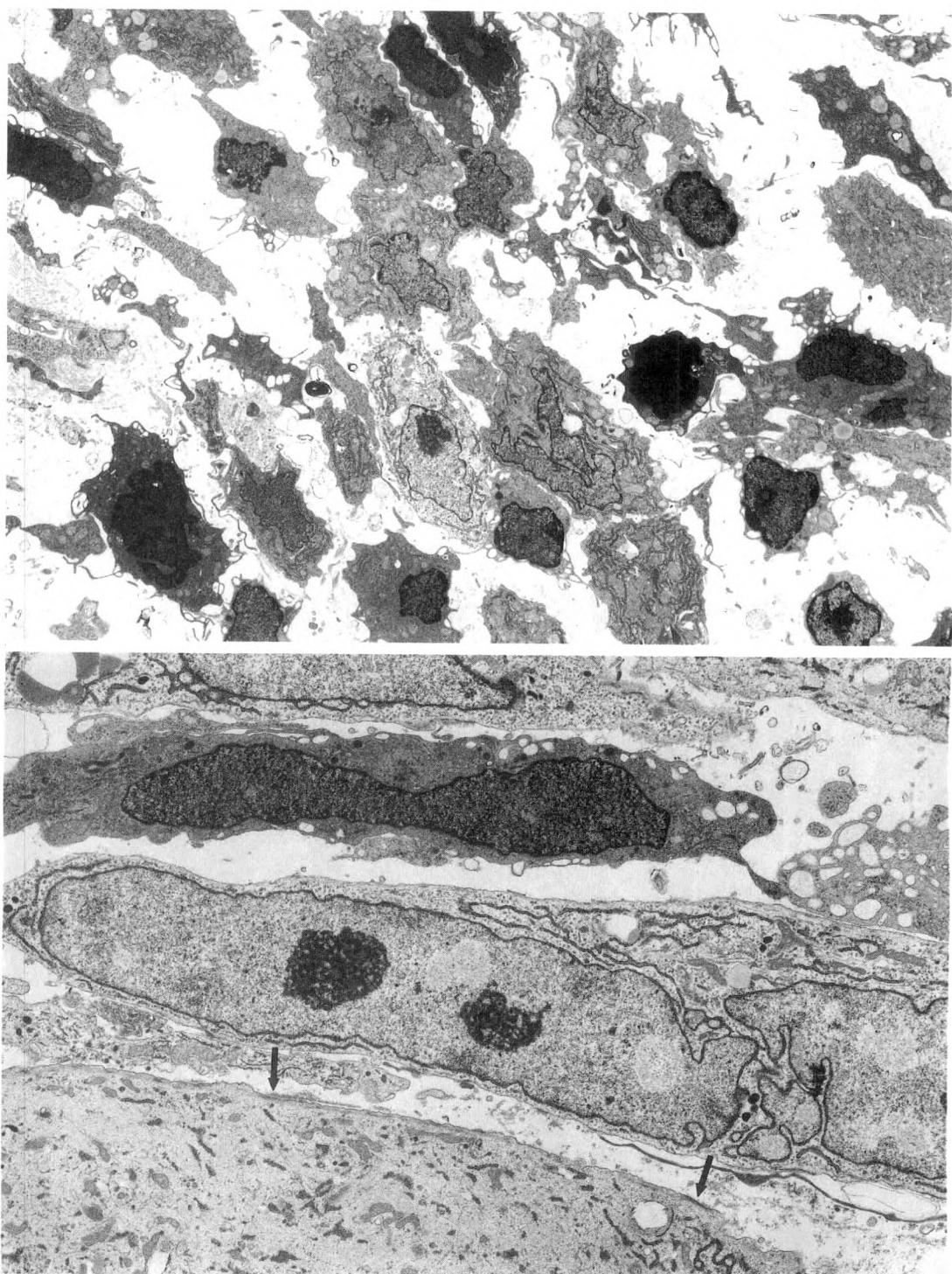
FIG. 5 (*upper*). Low-power electron micrograph shows spindle cells with moderately irregular oval nuclear profiles and cytoplasm containing abundant rough endoplasmic reticulum and prominent cell processes (×3,200).

FIG. 6 (*lower*). Higher-magnification electron micrograph of a more cellular area shows a subplasmalemmal collection of thin filaments at the periphery of some cells (*arrows*) (×6,000).

nodules)[7] occur from weeks to months after a surgical procedure and can be mistaken for a sarcoma because of their rich cellularity and brisk mitotic activity. PFMT, another benign genitourinary tract lesion, may histologically resemble postoperative spindle cell nodule but occurs in patients without a history of operative trauma. In 1980, Roth[9] first described PFMT, in a case involving the urinary bladder. Judging by the number of cases reported to date (see Table 1), PFMT seems to be rare. The prostate has been the site of involvement in only two reported cases,[3,10] and in one case the lesion was reported to be in bladder neck/prostate area.[11]

The exact histogenesis of PFMT is unclear, but its histologic features (the presence of granulation tissue-type vasculature and a prominent inflammatory cell component) and benign clinical evolution strongly suggest a reactive/pseudoneoplastic process. In the present case, as in previous reports,[8,10,11,13] immunohistochemistry and electron microscopy demonstrated a fibroblastic-myofibroblastic nature for PFMT.

Leiomyosarcoma and rhabdomyosarcoma are the malignant neoplasms that are most likely to be confused with PFMT because these lesions may have a myxoid histology. Because PFMTs are myxoid and may contain "strap" or "tadpole" cells, the chance of their being confused with rhabdomyosarcoma is especially great. However, the latter tumor characteristically shows condensation of tumor cells beneath the genitourinary epithelium (cambium layer), and constituent cells exhibit various stages of skeletal-muscle differentiation, with some showing cross-striation. The demonstration of skeletal-muscle differentiation in the tumor cells by immunohistochemistry and electron microscopy further establishes this diagnosis.

In adults, the most common sarcoma of the prostate is leiomyosarcoma. Most examples of this neoplasm are histologically high-grade tumors, with marked pleomorphism and a high mitotic rate. Pure myxoid leiomyosarcoma has not been documented in the prostate to our knowledge, although there have been reports of predominantly myxoid leiomyosarcoma in the urinary bladder.[15] Myxoid leiomyosarcomas tend to be less cellular, and mitoses are scanty. Hence, the differential diagnosis between PFMT and myxoid leiomyosarcoma may pose problems.[10] Strong immunoreactivity for smooth-muscle markers, together with ultrastructural evidence of muscle cell differentiation, can be helpful in achieving the correct diagnosis of leiomyosarcoma.

Other malignant neoplasms, such as neurofibrosarcoma, fibrosarcoma, and malignant fibrous histiocytoma, also should be considered in differential diagnosis of PFMT. However, in most cases the former tumors show sufficient cytologic atypia and mitotic activity, which prevent their being confused with benign lesions. Sarcomatoid carcinoma[16] also should be considered, particularly when only a small biopsy specimen is available. On occasion, this tumor may show focal myxoid changes, thereby creating confusion with other myxoid lesions, including PFMT. In our experience, sarcomatoid carcinoma is always a high-grade neoplasm;[16] therefore, a distinction with PFMT is straightforward.

Benign lesions of the prostate that may be confused with PFMT include postoperative spindle cell nodule and cystosarcoma phyllodes of the prostate. PFMT differs from postoperative spindle cell nodule with reference to the clinical history and, to some extent, histologic features. Postoperative spindle cell nodules usually are more cel-

TABLE 1. CLINICOPATHOLOGIC FEATURES OF REPORTED PSEUDOSARCOMATOUS FIBROMYXOID TUMORS OF THE GENITOURINARY TRACT

Reference	Age/Sex	Site	Mitotic Figures	Treatment	Follow-up Status
Roth, 1980[9]	32/F	Bladder	Frequent	Resection	AW/1 year
Hafiz and colleagues, 1984[3]	56/M	Prostate	None	Cystoprostatectomy	AW/1.5 year
Olsen, 1984[6]	24/F	Bladder	NR	Resection	NR
Nochomovitz and Orenstein, 1985[5]	73/F	Bladder	Rare	Resection	DOC
Nochomovitz and Orenstein, 1985[5]	22/M	Bladder	Rare	Resection	AW/3 year
Ro and colleagues, 1986[8]	56/F	Bladder	2/10 HPF	Partial cystectomy	AW/2 year*
Ro and colleagues, 1986[8]	52/F	Bladder	2/10 HPF	TUR	AW/3 mo†
Young and Scully, 1987[10]	59/M	Bladder	Occasional	TUR	AW/3.5 year
Young and Scully, 1987[10]	51/M	Prostate	None	TUR	R/2.5 year AW/8 year
Young and Scully, 1987[10]	59/F	Urethra	Occasional	Excision	AW/18 mo
Hughes, 1991[11]‡	47/F	Bladder	Occasional	Cystectomy	AW/9 yr
Hughes, 1991[11]	41/F	Bladder	Occasional	Resection	AW/4 yr
Hughes, 1991[11]	48/F	Bladder	Occasional	Partial resection	AW/2 yr
Hughes, 1991[11]	64/M	Prostate/Bladder neck	Occasional	Resection	AW/9 mo
Present report	22/M	Prostate	1/10 HPF	TUR	R/4 mo AW/4 year

NR = not reported; HPF = high-power fields; TUR = transurethral resection; AW = alive and well; DOC = died of other causes; R = recurrence.
* Latest follow-up: AW/6 year.

† Latest follow-up: AW/4 year.
‡ Hughes et al reported 5 cases and four of them are listed in this table. The fifth case is not included since the lesion appeared to be associated with an invasive transitional cell carcinoma.

lular than PFMTs and show a more compact arrangement of the spindle cells.[4] Furthermore, they tend to contain fewer inflammatory cells, and vascular proliferation in the stroma is not as prominent as in PFMTs.

Only a few cases of cystosarcoma phyllodes of the prostate have been reported.[17] Confusion with PFMT may arise when a small biopsy specimen contains predominantly the stromal component, and the typical biphasic pattern is not well seen. The vascular component and abundant inflammatory cells of PFMT discriminate between these lesions.

Although PFMTs are rare, as evidenced by the few cases in the literature, their accurate identification has great importance in avoiding unnecessary radical treatments. Because of the recent recognition and limited follow-up of PFMT, there is no universal agreement regarding therapy. However, management in all cases has consisted of either cystectomy or transurethral resection of the prostate or bladder. No patient has had metastases or died of disease. Our current recommendation is that conservative management, such as wide excision or partial resection, should be used, with close follow-up. When pathologists encounter this type of genitourinary tract lesion, clinicopathologic correlation is mandatory.

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Sex Cord Tumor with Annular Tubules Associated with Endometriosis of the Fallopian Tube

LINDA M. GRIFFITH, M.D., Ph.D., AND MARIA-LUISA CARCANGIU, M.D.

Sex cord tumor with annular tubules (SCTAT) is a distinctive ovarian sex cord-stromal tumor. The authors describe a case of SCTAT arising outside the ovaries, in the fallopian tube. The authors are aware of only one other case of extra-ovarian SCTAT, in an umbilical hernia sac in an adult woman. The case reported was an incidental finding in a hysterectomy specimen, obtained because of carcinoma *in situ* of the uterine cervix in a 32-year-old woman. The patient is alive and disease-free two years after surgery. The 1-cm tumor in the right fallopian tube was composed of typical ring-shaped tubules encircling hyalinized basement membrane-like material. In addition, glands and stroma of endometriosis were intimately admixed with the nests of tumor. The implications of these findings for the origin of SCTAT and ovarian sex cord structures are discussed. (Key words: Sex cord tumor with annular tubules; Gonadal sex cords; Endometriosis; Ovary; Fallopian tube) Am J Clin Pathol 1991;96:259–262

Sex cord tumor with annular tubules (SCTAT) is a rare form of ovarian sex cord-stromal neoplasm that was first described by Scully[1] in 1970. In patients with Peutz-Jeghers syndrome (PJS), these tumors usually are small, bilateral, and benign. Those occurring in females without PJS tend to be larger and unilateral,[2] and rarely are malignant.[2-6] Associated "adenoma malignum" of the cervix may occur in the absence of or more frequently in the presence of PJS.[2,7] SCTAT may arise in association with other ovarian tumors or exhibit foci of divergent differentiation.[2,5-12]

Two cases of SCTAT have been described in the testis of male patients.[13,14] The first extra-ovarian case of SCTAT in a female, arising in an umbilical hernia sac, recently was reported by Baron and colleagues.[15] We herein describe the second case of extra-ovarian SCTAT in a woman.

REPORT OF A CASE

The patient was a 32-year-old woman admitted to Yale-New Haven Hospital for total abdominal hysterectomy, bilateral salpingectomy, left oophorectomy, and appendectomy because of documented carcinoma *in situ* of the uterine cervix. The clinical history was negative for PJS,

and the patient had a right oophorectomy nine years previously. She is well two years after the diagnosis of SCTAT was made.

The operative specimen consisted of the uterus, right and left fallopian tubes, and left ovary. Both fallopian tubes exhibited multiple serous cysts. In addition, the mid-portion of the right fallopian tube was remarkable for the presence (on the serosal surface) of a 1-cm solid mass, which on cut section, was shown to consist of multiple minute, golden-brown nodules. These measured 0.3 cm in greatest dimension. The lumen of the tube was patent. The remainder of the specimen, including the appendix, was grossly unremarkable.

Microscopic examination of the fallopian tube mass revealed a multifocal tumor with the distinctive appearance of SCTAT, corresponding to the nodules that had been noted grossly. Many, but not all, of the characteristic epithelial nests, which were composed of ring-shaped tubules encircling hyalinized basement membrane-like material, in the lesion were intimately intermixed with foci of endometriosis (Fig. 1). Histologic continuity between areas of SCTAT and endometriotic stroma was noted; however, there was no direct continuity or merging of tumor nests and endometriotic glands (Fig. 2). The lesion closely approached, but was not continuous with, the overlying peritoneum. The serosa was composed of an intact layer of mesothelial cells with focal reactive change. Multiple foci of entrapped mesothelium also were present near the overlying peritoneal surface.

No evidence of SCTAT was identified in either the left ovary or left fallopian tube, which were examined histologically in their entirety. Microscopic examination of the uterus was unremarkable, except for mild dysplasia and endometriosis of the uterine cervix. Histologic material from the previous oophorectomy was available for review, and it showed a mucinous cystadenoma. In multiple sections of the latter specimen, there was no evidence of SCTAT.

DISCUSSION

The fallopian tube tumor described here represents, to the best of our knowledge, the second example of an extra-
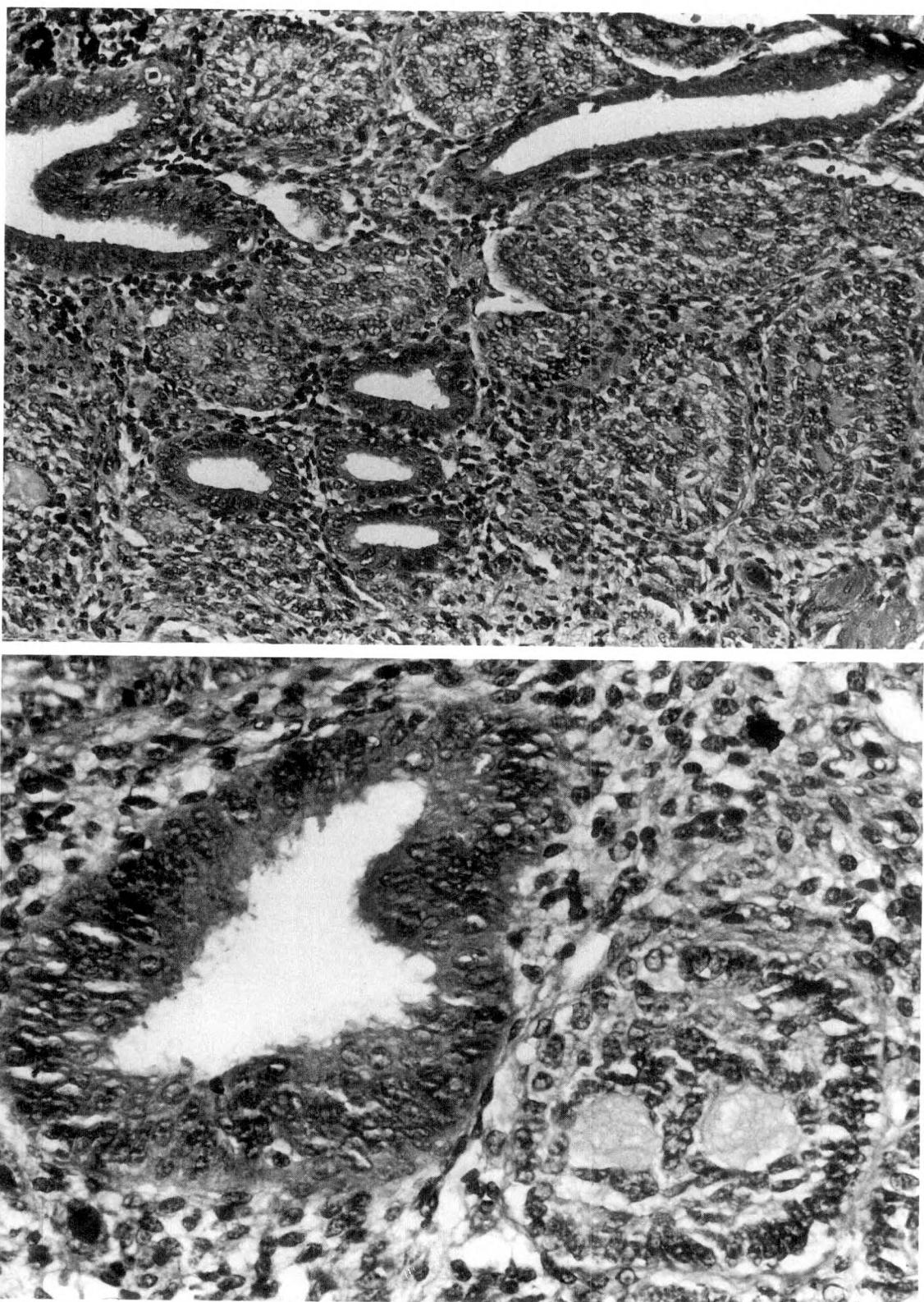
FIG. 1 (*upper*). Sex cord tumor with annular tubules, intimately intermixed with endometriosis. Clustered nests of columnar cells are arranged in a complex ring-like or annular pattern around central hyaline material. Typical glands and stroma of endometriosis are distributed randomly among the nests of SCTAT. Hematoxylin and eosin (×50).

FIG. 2 (*lower*). The distinctive histologic features of sex cord tumor with annular tubules are evident in this high-power photograph. The tumor nest approaches very close to the gland of endometriosis. Hematoxylin and eosin (×100).

ovarian SCTAT. The previously reported case was that of an incidentally discovered lesion in a ventral hernia sac of a 66-year-old woman without PJS.[15]

In our case, the possibility of metastasis from a primary ovarian neoplasm must be considered. Histologic review of the material from the ovary removed nine years previously revealed a mucinous cystadenoma as the only abnormality. Although we cannot exclude the possibility of microscopic foci of SCTAT having being missed in this specimen, we believe that this is unlikely. The association of SCTAT with mucinous cystadenoma is a rare event, which has been described only in patients with PJS.[8,11] Also against the hypothesis that the tubal tumor may represent a metastasis is the long interval of time between the original oophorectomy and the recent surgery. Furthermore, if a malignant SCTAT had been present in the right ovary, one would have expected an extensive tumor burden currently. Finally, it would seem most unusual for a neoplasm to metastasize exclusively to a focus of endometriosis. We have seen malignancies that spread to an area of endometriosis, but they usually occurred as part of widespread dissemination.

Granulosa cell tumor or Sertoli-cell tumor constitute, in many cases, a prominent conjoint component with SCTAT,[2,6,10,12] suggesting a potentially shared origin for these lesions.[16,17] Immunohistochemical studies have shown that SCTAT, as well as granulosa cell and Sertoli-cell tumors, can be reactive for keratin and vimentin.[18] At present, SCTAT is regarded as a distinctive sex-cord-stromal tumor with the potential for bidirectional differentiation.

The existence of two cases of SCTAT that appear to have arisen outside the ovary necessarily elicits considerations of the origin of these tumors, and consequently, of the derivation of the ovarian sex cords. Whereas it is widely accepted that primitive germ cells migrate to the gonads from the yolk sac, the origin of the sex cords that invest the germ cells within the gonad is less certain. Classic theory holds that the "germinal epithelium" (coelomic epithelium), which covers the ovarian surface, grows inward to surround the germ cells. There may be one or two "sproutings" in this process.[19–21] The sex cords would thereby originate from the "germinal epithelium." The currently favored hypothesis suggests that the germ cells induce sex cord differentiation in the ovarian mesenchyme that surrounds them. Therefore, the coelomic epithelium would not be involved in the formation of the sex cords.[21,22] If one accepts the first of these theories, it can be postulated that the coelomic epithelium could give rise to a sex-cord-type tumor in adults. Thus, this case and the one reported by Baron and colleagues may have had their origins *de novo* in the coelomic epithelium.

It also is remarkable that in our case, foci of endome-

triosis were intimately admixed with the neoplastic proliferation. Because the most accepted theory for the origin of peritoneal pelvic endometriosis proposes that the pelvic mesothelium undergoes müllerian metaplasia into endometrial-type tissue, it is possible that both the SCTAT and the adjacent endometriosis may have originated from the coelomic epithelium.

Other examples of extra-ovarian sex cord tumors have been described, but these were all pure granulosa cell tumors. These cases have been interpreted by the authors as arising from ectopic ovarian tissue.[23–25]

Another alternative that needs to be considered is that the tumor we have described represents an example of so-called sex cord-like tumor arising within a focus of endometriosis. Examples of this entity have been described by Clement and Scully[26] in the uterus, either in a pure form (type II) or in association with endometrial stromal sarcoma (type I). Young and colleagues[27] have described nests of cells that simulate ovarian sex cord elements in seven cases of stromal sarcoma of the ovary. In uterine and ovarian sex-cord–like neoplasms, the constituent cells usually are arranged in cords and tubule-like structures, simulating Sertoli-cell tumor and granulosa cell tumor. However, they do not appear to be able to form structures that are similar to the characteristic ring-shaped tubules of SCTAT. Immunohistochemistry is not useful in distinguishing sex cord tumor (and, in particular, SCTAT) from sex-cord–like tumor because both are positive for keratin and vimentin.[18,28]

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An Autopsy Case of I-Cell Disease

Ultrastructural and Biochemical Analyses

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An autopsy case of I-cell disease in a 4-year-old Japanese girl is presented. In this report, the authors analyze the relationship between morphologic (including electron microscopic) and biochemical findings. Lymph node, spleen, and kidney, which were stained with Hale's colloidal iron method, contained large amounts of hexosamine. These substances had accumulated in lymphocytes of B-cell lineage. (Key words: Metabolic storage diseases; Lysosomal disorders; Mucolipidoses) Am J Clin Pathol 1991;96:262–266

I-cell disease (mucolipidosis II), a rare metabolic disorder, was first described in 1967 by Leroy and DeMars.[1] They noted coarse cytoplasmic inclusions in fibroblasts cultured

from the skin of an affected child. I-cell disease is characterized by intracellular lysosomal enzyme deficiencies leading to accumulation of UDP-Glc-NAc, a substrate of the enzyme Glc-NAc-1-P transferase.[2] The latter catalyst is required for the transport of acid hydrolases into lysosomes but is defective in I-cell disease. Biochemical analyses of affected tissues have revealed an accumulation of mucopolysaccharides,[3] lipids,[4] and oligosaccharides.[5,6] Histopathologic studies have demonstrated that the most consistently affected cells include fibroblasts, pericytes, endothelial cells, spinal anterior horn cells, Schwann's

cells, renal podocytes, and renal tubular cells.[2,7-9] Clinically, I-cell disease resembles Hurler syndrome and is manifested by growth and psychomotor retardation, coarse facial features, and severe skeletal changes. The pathogenesis of I-cell disease is not fully understood. This report describes the autopsy findings in a 4-year-old girl with I-cell disease. Special reference is made to ultrastructural and biochemical analyses.

REPORT OF A CASE

A 2-month-old girl, who had been born by normal delivery at 40 weeks' gestation, was brought to the doctor because of "feeble crying." Biochemical analyses revealed that serum concentrations of lysosomal enzymes (excluding beta-glucosidase) were remarkably high. Chest roentgenograms showed oar-shaped deformities of the ribs, scoliosis, and abnormalities of the spinal column. Psychomotor retardation was conspicuous. These findings strongly suggested a diagnosis of I-cell disease, although cutaneous fibroblasts were not cultured for the identification of cytoplasmic inclusions. She experienced repeated respiratory infections and underwent home oxygen therapy because of chronic respiratory failure. The child died of respiratory failure on March 31, 1988.

Consanguinity did not exist in the family, and the child's mother had no history of stillbirths. The patient was her fourth child. The first child died of respiratory failure 23 days after birth, and the second and third children are healthy with no psychomotor retardation. There is no history of I-cell disease on either the maternal or paternal sides of the family.

IMMUNOHISTOCHEMICAL AND HISTOPATHOLOGIC FINDINGS

Pertinent histologic and immunohistologic findings at autopsy were found in several organs and are described here.

Spleen

Histiocytes in the marginal zone of the splenic parenchyma were remarkably swollen and stained with Hale's colloidal iron method (Fig. 1A). The cytoplasm of resident lymphocytes also was colloidal iron-positive but less markedly so.

Lymph Nodes

Lymphocytes in the cortical follicles were labeled by Hale's colloidal iron (Fig. 1B). These cells were found to be B lymphocytes by immunohistochemical studies using a monoclonal antibody against a CD22 antigen (DAKOPTTS A/S Copenhagen, Denmark).

Thymus

Thymocytes also were stained with Hale's colloidal iron (Fig. 1C), although the reaction was weak. There were no abnormalities in the thymic epithelium.

Heart

Fibroblasts in the myocardial interstices were stained with Hale's colloidal iron. The myocytes showed no inclusions in the cytoplasm.

Kidney

Epithelial cells (but not mesangial cells or endothelial cells) of the glomeruli were vacuolated and stained with Hale's colloidal iron technique (Fig. 2).

Other Organs

There were no significant changes in other organs, except for the lungs. They showed organized and acute pneumonia, representing the immediate cause of death.

ULTRASTRUCTURAL EXAMINATION

Small pieces of several organs were fixed in 2% glutaraldehyde containing 0.1 M phosphate buffer (pH 7.4), followed by immersion in osmium tetroxide. After dehydration with graded ethanols, they were embedded in Spurr® low-velocity embedding media (Polysciences Inc., Warrington, PA). Ultrathin sections were cut on a Porter-Blum ultramicrotome (Sorvall Inc., Connecticut) and observed with a Hitachi H-600® electron microscope (Hitachi, Ibaragi, Japan) after counter-staining with uranyl acetate and lead citrate.

Splenic lymphocytes contained cytoplasmic vacuoles of varying sizes, as did lymph node lymphocytes. The latter were identified as B cells because of an abundance of rough endoplasmic reticulum (Fig. 3A). Thymocytes likewise were vacuolated; also, in contrast to light microscopic findings, the thymic epithelium was found to harbor inclusions (Fig. 3B). Fibroblasts of the myocardial stroma, renal podocytes, hepatic Kupffer's cells, and acinar pancreatic epithelium also exhibited the presence of cytoplasmic vacuoles.

BIOCHEMICAL ANALYSIS

Tissue or organs (0.2–0.4 g wet weight) that had been stored at −80 °C were thawed and washed with saline at room temperature. The samples were then homogenized immediately on ice, using an Ultra Turrax T-25® (Jankel and Kunkel, Stanfen, Germany) twice for 1 minute with 4 mL of 0.01 M phosphate-0.15 M NaCl buffer (pH 7.4). Aliquots of the homogenate were used to determine protein concentration and phospholipid content. The protein concentration was measured by the method of Lowry and colleagues,[10] using bovine serum albumin as a standard. To measure phospholipid content, the homogenate (1 mL) was extracted using the method of Bligh and Dyer.[11] Chloroform (1.25 mL) and methanol (2.5 mL) were added
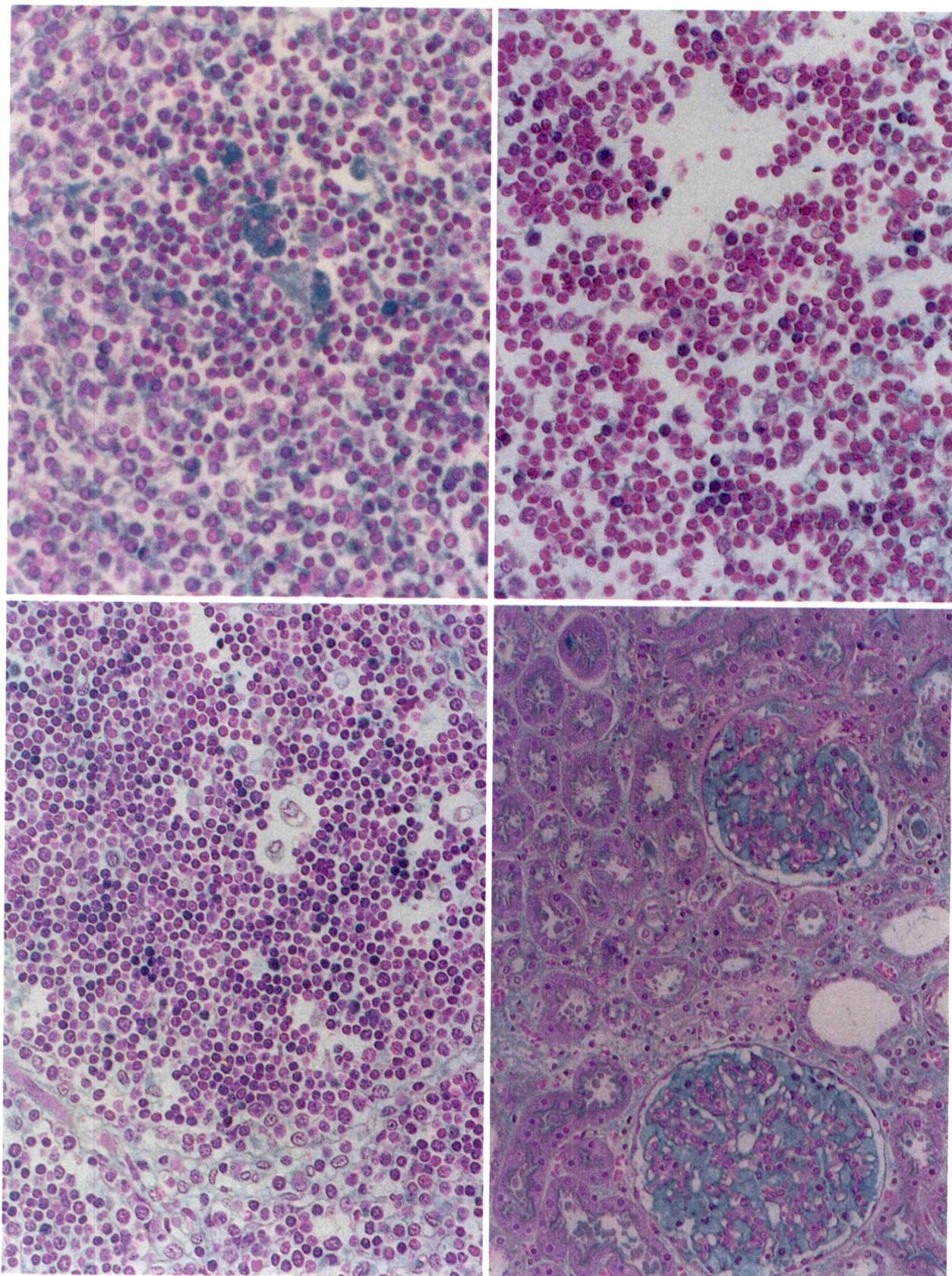
FIG. 1. *A* (*upper, left*). Splenic histiocytes stained with colloidal iron.

FIG. 1. *B* (*upper, right*). Lymphocytes and histiocytes in the lymphoid follicles stained with colloidal iron.

FIG. 1. *C* (*lower, left*). Thymocytes as well as epithelial cells showing weak positive staining with colloidal iron.

FIG. 2 (*lower, right*). Swollen epithelial cells of the renal glomerulus stained with colloidal iron.
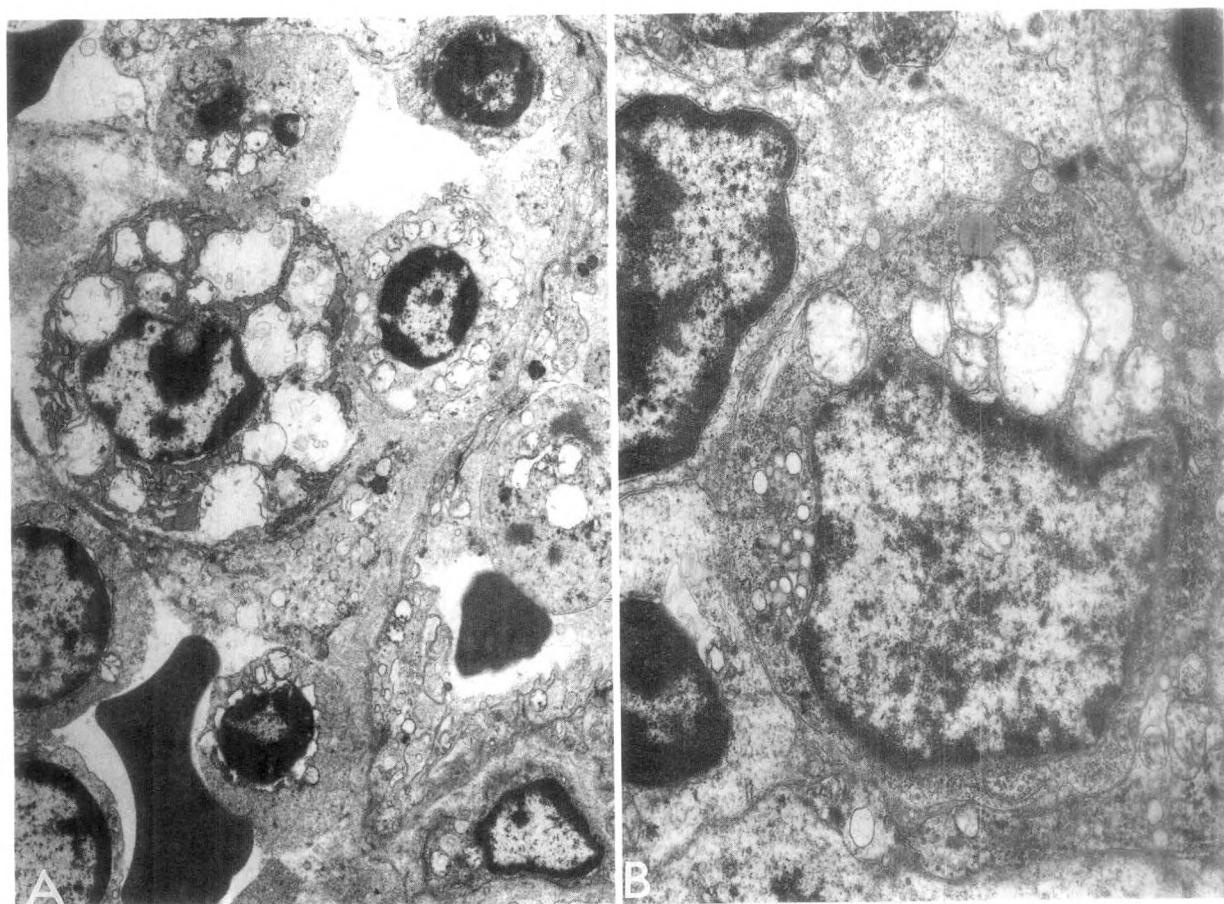
FIG. 3. *A* (*left*). Lymph node B cells with many vacuoles in the cytoplasm. *B* (*right*). Thymocytes and epithelial cells with vacuoles in the cytoplasm.

to the homogenate, mixed, and centrifuged ($1,600 \times g$ for 5 minutes). The supernatant was mixed with chloroform (1.25 mL) and methanol (1.25 mL), and centrifuged. The resulting chloroform layer was dried using nitrogen, and the lipid-phosphorus content was determined using the method of Bartlett.[12] The residual homogenate was centrifuged ($10,000 \times g$ for 15 minutes). According to the Orcinol-H_2SO_4-reaction[13] and Elson-Morgan reaction,[14] the neutral sugar and amino sugar contents of the supernatant were determined, using glucose and glucosamine

as standards, respectively. In the latter method, the supernatant was pretreated with 2 N HCl at 100 °C for 15 hours and used for the assay. As shown in Table 1, the amounts of tissue hexosamine were highest in the lymph nodes, spleen, and kidney.

DISCUSSION

Hasilik and Neufeld[15,16] found that cultured skin fibroblasts from patients with I-cell disease showed increased activity of three lysosomal enzymes (β-hexosaminidase,

TABLE 1. CHEMICAL CONTENTS OF ORGANS IN I-CELL DISEASE

Organ	Hexose (μmol)			Hexosamien (μmol)			Phospholipid (protein) (μmol/mg)	Phospholipid (μmol/g)	Protein (mg/g)
	/g (w.w.)	/Phospholipid (μmol)	/mg protein	/g (w.w.)	/Phospholipid (μmol)	/mg protein			
Muscle	4.23	0.4	0.03	1.84	0.20	0.01	0.07	9.06	129
Cardiac muscle	1.53	0	0.01	4.30	0.17	0.03	0.20	25.5	127
Thymus	1.50	0.2	0.02	2.89	0.39	0.03	0.08	7.37	97.1
Lymphnode	2.33	0.1	0.02	5.72	0.39	0.06	0.15	14.5	95.1
Liver	2.71	0	0.02	4.03	0.11	0.03	0.24	35.7	149
Spleen	2.91	0.1	0.03	6.84	0.35	0.08	0.22	19.5	90
Kidney	2.01	0	0.02	6.85	0.32	0.06	0.17	21.3	123

w.w.: Wet weight.

α-glucosidase, and cathepsin D) in the culture medium. In addition, they observed that the enzymes were not phosphorylated, indicating defective "recognition markers" in these lysosomal catalysts. Glycoprotein metabolism presumably is inhibited because of low activity of such enzymes in I-cell disease.

Biochemical analyses in the present case revealed that hexosamine was abundant in the lymph nodes, spleen, and kidney (Table 1). It is well-known that peripheral lymphocytes in I-cell disease contain prominent intracytoplasmic vacuoles. In the present case, many lymphocytes in the thymus, spleen, and lymphoid follicles of the lymph nodes were labeled with Hale's colloidal iron. In the spleen, intracytoplasmic vacuoles have been detected in reticuloendothelial cells, cells in germinal centers, the adventitial cells of blood vessels, and sinus histiocytes. Macrophages stained with Hale's colloidal iron were observed in the spleen in our case, but there were fewer positive lymphocytes. In the lymph nodes, many lymphocytes were stained.

Using an immunohistochemical technique, we found that the colloidal iron-positive lymphocytes in the germinal centers were B lymphocytes. Vacuole formation was recognized ultrastructurally in organelles that we believed to be mitochondria. The same findings were noted in plasma cells, which have abundant endoplasmic reticulum. In the thymus, epithelial cells showed multiple vacuoles, whereas thymocytes had few. It should be noted that the substances that were stained with Hale's colloidal iron had accumulated in lymphocytes of B-cell lineage rather than in those of T-cell lineage. At present, the mechanisms and implications of this phenomenon are unknown and merit further study.

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Special Report

Assuring Continued Competence

Is Recertification the Way?

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The American Society of Clinical Pathologists conducted the second of its series of invitational conferences* on current concerns of the profession at Colorado Springs, Colorado, May 4–6, 1990.[1] The theme was Assuring Continued Competence, and a challenging question concerned whether there should be a recertification program in pathology.

Almost 20 years previously, the American Board of Family Practice was approved by the American Board of Medical Specialties (ABMS) with a provision that all certifications would be for a limited period of time and individuals would have to renew their certifications periodically. The American Board of Pathology (ABP), in concert with a number of other boards, explored the issue in the early 1970s and was authorized by the ABMS to initiate a program of recertification. After further consultation with its constituents and supporting societies, the ABP suspended plans for a program, although the topic has repeatedly surfaced in its deliberations. Lately, societal and legal attitudes and activities by the federal government and the state of New York, among others, have intensified general concern about the topic.

Invitations to the conference were extended to officers of national and state pathology societies, departmental chairmen and program directors of medical schools and teaching hospitals, officers of the ASCP, persons known to be involved in setting credentials and certification of pathologists, and other individuals recognized to be interested in the topic. Participation in the various stages of the conference varied slightly, but of the 143 individuals who responded to a demographic survey, 91% were physicians; 56% were younger than 55 years; 77% were certified in anatomic pathology/clinical pathology; 68% identified their type of employment as "service laboratory"; 53% worked in university or university-affiliated hospitals and 29% in nonuniversity hospitals.[2] The final group of participants supplied a broad base of opinion from an expert, informed, and concerned group of somewhat self-selected leaders of clinical pathology in the United States.

ORGANIZATION OF THE CONFERENCE

To extract a maximum amount of information from the participants, the Program and Planning Committees* decided to employ two group techniques to achieve maximal results: the Delphi and the Nominal Group processes. The Delphi would sample opinions held before, during, and at the end of the conference. The Nominal Group would be used to generate ideas that could be shared with the full conference group and that might clarify and influence the evolution of opinions during the conference. Both techniques[3] would allow the expression of opinion and position by every individual and would yield a sort of quantitative expression of the values placed by the group or subgroups on particular ideas. Four iterations were executed for the Delphi, which was composed of 95 items that were not changed during its course. The Nom-

inal Groups were constituted so as to accommodate the large attendance and were compressed within time schedules that somewhat inhibited the extended exploration of meanings and congruences.

Most of the attendees seemed to have had some experience with the Delphi technique but little with the Nominal Group. Several expressed discomfort with the restrictions of free and unlimited expression during Nominal Group sessions; nevertheless, participation was at least complaisant if not enthusiastic.

Four iterations of the Delphi were performed: two before the conference began, a third after most of the plenary addresses and participation in the Nominal Group procedures, and a final after a concluding session of free discussion. Each participant took part in two Nominal Groups that addressed topics related to the announced objectives of the conference.

PLENARY ADDRESSES AND DISCUSSIONS

Dr. George D. Lundberg opened the presentations by emphasizing the increased criticism from the literary media and public that addressed the medical profession in general and pathology in particular. He offered a quotation: "For better or worse, for richer or poorer, in sickness and in health, 'til death do us part," and asked if that familiar statement describes modern American marriage. "Certainly not. Approximately 50% of such end in divorce, often very early. What it does describe is licensure to practice medicine and surgery in nearly any of these United States and certification by the ABP after entry-level examination—lifelong and forever. Should that be?" He went on to emphasize current reactions to defects in laboratory performances and legislative and regulatory invasions of the practice of pathology, particularly the Health Care Financing Administration's plans regarding cytopathology.

In consideration of the nature of competence, Dr. David Smith emphasized that its most significant measurement was by surveillance and evaluation of its manifestations (*i.e.,* performance), over time rather than in episodic mechanized tests of knowledge. Although the latter undoubtedly are the most efficient measurement tools available and are appropriate for entry-level qualification, they are deficient in relevance and inherently weak for predictive validity of actual practice. Nevertheless, a recredentialing program, based on evaluating performance rather than by a challenge examination, would require a considerable effort at education of the public and particularly of politicians, who are most likely to make the determinative decisions regarding its implementation.

Since the conference the description of a plan for periodic physician recredentialing that is being developed

in New York has been published. It proposes prospective, not retrospective, evaluation by peers of compliance with defined standards of practice.[4]

Of the 23 primary specialty certifying boards of the ABMS, 19 have approved programs for recertification, and 17 of these place time limitations on the validity of all newly issued certificates. No board imposes mandatory recertification on diplomates who have been issued an originally unlimited certificate. The program of the ABP is largely inactive. It has provided new certificates only to diplomates who needed such for licensure by endorsement because of requirements by several states that prior examination be no earlier than a specified number of years. These new certificates were issued to candidates taking and passing a current primary certification examination. To date, all pathologists who have had to use this route have been successful. The requirement that a cognitive examination be included in any recertification procedure was made a condition for approval of the program by the ABMS after rejection in 1977 of a proposal based on evaluation of manifestations of competence without repetition of a knowledge examination.

Dr. Jack P. Strong, president of the American Board of Pathology in 1990, described several consensus statements of the Board:

1. The American Board of Pathology is the only body authorized by the ABMS to certify pathologists and thus the only body that can recertify these pathologists.
2. Recertification of pathologists can be available only to holders of primary certification certificates. Initial endeavors in recertification should be restricted to the area of primary certification.
3. The American Board of Pathology has a responsibility to its diplomates who desire recertification.
4. The process of recertification must be educational and meaningful.
5. Continuing medical education and practice assessment should be major components of any recertification process.
6. The ABP should not be involved in recredentialing noncertified pathologists. Other pathways should be used.

In addition, the Board cannot require that holders of its certificates be recertified.

He further presented the following policy statements:

1. The American Board of Pathology opposes time-limited and mandatory recertification.
2. The American Board of Pathology approves the concept of voluntary recertification as one pathway for the recredentialing of competence.
3. Voluntary recertification for the holder of a primary

certificate may be accomplished by some combination of continuing medical education, assessment of practice performance, a cognitive examination, and possibly other approaches.

4. The American Board of Pathology will not supply lists of recertified pathologists for publication.

Dr. Harris B. Graves, past president and the current chairman of the Recertification Committee of the American Board of Emergency Medicine described how that Board has worked with an established academic testing group and is now in the process of developing a recertification procedure. In both the certification and recertification procedures there is a strong effort to evaluate the manifestations, as well as the elements, of competence. Chart Stimulated Recall (CSR), an evaluative procedure that uses an examinee's actual cases, is an innovative approach. Several varieties of written examination (at-home, closed- and open-book, and domain-specific) have been employed in field tests and have proved reliable and valid, as has the CSR. Certification scores are strong predictors of recertification scores, with 7% gaining points, 7% losing points, and 95% passing. This experience with the predictive value of the original certification scores was particularly intriguing to the participants in the conference.

The process by which the American Board of Anesthesiology and the American Society of Anesthesiologists is developing a program for assuring continued competence was described by Dr. D. David Glass. Its goal is a voluntary program that will be referred to as "Continued Demonstration of Qualifications" (CDQ). The approach is based philosophically on a concept that their primary certification is for consultant-level qualifications and not minimal competence, which does little to meet the needs and problems of uncertified practitioners. Because of the inherent character of the practice of anesthesiology, strong emphasis is placed on personal behaviors, especially in relation to chemical dependency.

The development of the National Practitioner Data Bank was described. Information regarding specialty certification is not included, but inclusion of reports from specialty boards on revocation of certificates (not failure on examinations) could grant the boards legal immunity in disciplinary actions involving diplomates.

The Federation of State Medical Boards operates an extensive central file on disciplinary actions by its component boards. This embraces large numbers of problem physicians. The approach considered realistic is to focus on the deficient individuals and sharpen methods of detecting, correcting, and rehabilitating them, rather than subjecting the vast majority of competent physicians to exercises for confirmation of the manifestations of competence they regularly practice.

From a legal standpoint, certification by voluntary organizations within the profession has led a charmed life, but challenge under the antitrust laws, which are exclusively interested in fostering competition, has always been possible and very likely would be encouraged if a recertification procedure were to take away important work credentials from practicing physicians. The crucial item is the detection of and dealing with incompetent physicians, not the assurance of continuing competency among those who are practicing competently. Due process procedures must be followed. It will be important to find liability protection for those who are responsible for judgments in any program of mandatory recertification. Voluntary recertification eventually would be required *de facto* by credential committees and under insurance payment schemes, just as primary certification has become so essential for first-class practice opportunities.

Dr. Jerald R. Schenken raised questions as to whether the system was broken and in need of fixing. Pathology already has more programs by national societies that survey and accredit practice and give continuing education to pathologists than does any other specialty. May not the problem be in convincing others that such are, or with improvements can be, the best way to assure continuing competence through consideration of its manifestations? The cost, in the time and effort of professionals, as well as out-of-pocket expenditures, for participation in present programs is considerable. The effective cost of continuing education for family physicians attending the review course at the University of Nebraska is on the order of $10,000–15,000 for a six-year cycle of recertification. These expenses inevitably are reflected in increased costs of medical care. With programs of rationing medical care on a cost basis being considered in Oregon and by other governmental agencies, employers, and insurers, can the profession in good conscience add more expenditures in the name of predictive validity that is unproven, if not impossible?

The most challenging and fundamentally innovative concept presented during discussions implied that the problem before the conference should be the detection and correction of incompetence rather than the assessment of continuing competence. Dr. George Stevenson pointed out that physicians in the United States are almost unique in their concern with the reaffirmation of their competence. Elsewhere credentials, once obtained, are assumed to indicate competence unless individuals disqualify themselves. The detection of incompetence, however, cannot be expected by employing the approaches used to maintain or demonstrate competence, especially since unknown hundreds of pathologists practice without ever having passed the ABP's examination. Entirely new methods should be developed.

In the plenary addresses it was mentioned that present evaluations of the elements of competence are based on examinations that objectively demonstrate incompetence at the time of the examination and, despite many aspirations to the contrary, can never reach absolute, or even very statistically satisfactory, predictive validity for future expressions of the manifestations of competence.

The licensing boards, dealing with the large numbers of impaired and otherwise incompetent physicians, approach their responsibilities by focusing on the deficient, rather than the effective, practitioner. Several speakers asserted that the "outliers," or the uncertified pathologist, or the pathologist who does not participate in various quality assurance activities of professional societies are likely the most important concern. The responsibilities of any program to eliminate the incompetent practitioner are serious and raise the specter of liability for restraint of trade, but protection for persons who assume such responsibility has been developed in the areas of peer review organizations and the National Practitioner Data Bank.

Discussions also involved a considerable interest in the report of the American Board of Emergency Medicine that performance on the original certification was the best predictor of performance on recertification. It was suggested that at some score on the original process the examinee might be given an unlimited certification, whereas those with lower scores would receive time-limited certificates.

Other comments during discussion periods, particularly those following the reports of Nominal Group section leaders and some of the plenary addresses, were appropriate extensions of the topics of those reports and addresses. Concern with the uncertified practitioners, the pathologists who do not participate in activities of the societies, and the otherwise impaired pathologists was expressed and emphasized as a problem. There were varied comments and estimates regarding the numbers of such individuals, with the possibility of there being 3,500 or more. Mandatory recertification, in addition to being generally rejected by the audience, was recognized as a particularly impossible problem in dealing with most such individuals.

Several speakers presented resolutions or surveys, formal and informal, from pathology societies or groups that expressed strong opposition to any form of recertification, although some added qualifications that should be sought if a process were imposed. Nevertheless, participant opinion as expressed in the Delphi, somewhat in contrast to the speeches in the discussions, remained at about a two-thirds majority considering recertification as a good thing and was 95% for acceptance of voluntary recertification, with a strong recommendation to the ABP to initiate a program.

The limitations on free early discussion imposed by use of the group techniques for most of the conference were criticized. Concern that numerically expressed outcomes would be considered binding resolutions were expressed, although others emphasized that the techniques were for the broad elicitation of ideas and opinions from each individual participant.

PREPONDERANT OPINIONS FROM THE DELPHI

Because of the size and nature of the audience it was necessary to modify slightly the usual protocol for the Delphi technique. The roster of participants was completed shortly before the beginning of the conference, so it was not possible that all could participate in generation of the questions of the Delphi. Instead, members of a select committee composed 95 questions that they believed would allow all to express their positions regarding many facets of the objectives of the conference and would provide intellectual stimulation. Subjects and responses ranged from 100% acceptance (marred by only one undecided vote) for, "If recertification comes to exist for pathologists in the United States, multiple pathways should be available in order to fit the varied practice patterns of pathologists," to 89% rejection (77% strongly so) with 7% no opinion expressed regarding a whimsical quotation from George Bernard Shaw, "All professions are a conspiracy against the public."

The numbers of registrants that participated in the four repetitions of the Delphi are shown in Table 1.

The four passes displayed a gradual solidification of opinion without dramatic shifts. The average *No Opinion* vote fell from 6.0% in the first trial to 2.7% in the last. Items approved or disapproved by 85% or more progressed from 15 to 45, while those that were approved or disapproved by between 40% and 60% of participants fell from 30 to 11 (Table 2). Only one item changed from approval to disapproval and three from disapproval to approval by the majority. None of those moved into the 85% category.

A detailed rating scale analysis was performed to demonstrate the strength of acceptance or rejection and to compare overall patterns of change for each item, the latter calculation reflecting achievement of consensus (ME Lunz, summary report of the Delphi study). This made

TABLE 1. NUMBERS OF REGISTRANTS THAT PARTICIPATED IN THE FOUR REPETITIONS OF THE DELPHI

Iteration	*Number Responding*
1	136
2	119
3	116
4	109

TABLE 2. CHANGES IN DELPHI RESULTS
THROUGH FOUR REPETITITIONS

Delphi Results	Pass 1	Pass 2	Pass 3	Pass 4
Number of items approved or disapproved by 85%	15	31	37	45
Number of items approved or disapproved by 40–60%	30	17	14	11
Average "No Opinion" per item	6.0%	3.6%	2.8%	2.7%

it clear that most participants felt that: (1) pathology societies should provide courses and publications to assist practicing pathologists, (2) if recertification were required, sought and failed, redemptive educational programs to encourage achievement of recertification should be available, and (3) multiple pathways for recertification to fit varied practice patterns should be available.

The attributes of recertification most approved were:

1. any recertification should include a practice component;
2. it should be tested on a trial basis to determine its effect before it is implemented;
3. the cost should not exceed the cost of initial certification;
4. costs should be paid by the individual physician through fees.

Acceptable pathways to recertification in order of preference were: (1) continuing education courses; (2) laboratory accreditation results; (3) examinations in specialty areas; (4) evidence of performance on surveys; (5) audits of selected cases or charts; (6) evidence of patients' outcomes; (7) results of proficiency surveys; and (8) evidence of professional liabilities or malpractice actions. Retaking the initial American Board of Pathology examination as a method of recertification was disapproved by most participants.

Analysis of the magnitude and direction of change for various items provided insight into the extent of developing consensus and the effects of the course of the meeting and of the Delphi. The five questions that showed by weighted analysis the most significant gains in approval were:

1. Some reasonable method to assess the behavioral/alcohol/other drug-impairment aspects of pathologists' practice performance should be developed and applied in both relicensure and recertification.
2. Any recertification process should be implemented on a trial/study basis only, until data have accumulated to determine whether it has had an impact on patient care.
3. The cost of recertification should not exceed the current cost of certification.

4. Recertification should include a practice assessment component.
5. If relicensure or recertification becomes required, professional pathology societies should provide courses and publications in a deliberate and organized effort to assist the practicing pathologist in this effort.

The five items that exhibited the most significantly increased disapproval, with the greatest listed first, were:

1. All professions are a conspiracy against the public, as George Bernard Shaw alleged.
2. Neither initial board certification nor state licensure nor subsequent recertification nor relicensure are necessary.
3. A free marketplace should evaluate professional competence and let the buyer (patient) beware.
4. Recertification of pathologists would improve patient care in the United States.
5. State Boards of Medical Examiners should limit licenses to specific areas of physician training/specialty/interests or expertise.

CONCLUSION

Results of the Delphi and Nominal Group techniques, as well as summaries of the plenary addresses and discussions, have been published in extended form.[2] In general, there was a consolidation and intensification of support or rejection of various ideas, but no substantial reversals occurred. It was noteworthy that, although there was much spontaneous reluctance to accept the term "recertification," the concept of assuring continuing competence was supported and most technical suggestions for its implementation were recognized as possibly appropriate in one or another application.

The most strongly supported ideas were:

1. A voluntary, and not a mandatory, recertification program should be made available by the American Board of Pathology.
2. Any contemplated recertification program should give primary attention to the manifestations of competence by use of practice surveys, laboratory accreditation, etc.
3. Any contemplated recertification program should be adjusted for the variety in the manifestations of competency practiced by pathologists.
4. Pathology societies should develop and offer educational programs that would prepare a pathologist for recertification or aid the correction of deficiencies revealed during recertification.
5. Some reasonable method to assess the performance of impaired pathologists should be developed and applied in any contemplated relicensure or recertification.

ADDENDUM

The ASCP Board of Directors adopted the following policy at its meeting June 23, 1990.[5]

Preamble

The role of pathologist recertification in assuring continued competence in the practice of pathology is controversial. The subject has been debated for several years in various pathology organizations including the American Society of Clinical Pathologists (ASCP). A consensus position on recertification has not evolved. Presently there is no formal recertification program available for pathologists in the United States. In this respect, pathology stands with only five other members of the American Board of Medical Specialties. Seventeen specialty Boards presently offer some form of recertification process for their diplomates, although there are no convincing data to demonstrate that recertification improves medical practice.

The ASCP Board of Directors is rightfully interested in the issue of recertification since we are primarily an educational association concerned with the continuing education of pathologists and advancing the quality of practice of pathology and laboratory medicine in America. Assurance of competence in pathology practice is inherent in the mission statement of the ASCP.

Although there are presently no legislative mandates nor state medical board requirements for specialty recertification, there are several factors that have influenced the Society's decision to develop a policy statement. These include:

1. Present requirements for initial licensure in some states that include documentation of recent success in passing a specialty board or FLEX examination.
2. Potential for state licensure requirements for periodic recertification.
3. Potential for a recertification requirement for reimbursement for pathology professional services from Medicare and other third-party payers.
4. Potential for recertification requirements for hospital privileges and credentialing.
5. The perception that initial certification does not guarantee lifelong competence.
6. Most other medical specialties' Boards offer recertification on a voluntary basis or issue time-limited initial certification.
7. The need to plan and promote a recertification process that is most likely to assess competence and be pal-

atable to the profession in the event of government-required recertification.
8. A need for the ASCP to plan and provide courses and materials to assist pathologists in preparing for a recertification process.

POLICY

• The ASCP does not support *mandatory* recertification or time-limited initial certification for pathologists.

• A *voluntary* recertification process should be made available for diplomates of the American Board of Pathology who have need for it.

• The American Board of Pathology should construct a multifaceted voluntary recertification process that includes multiple pathways in order to fit the varied practice patterns of pathologists.

• The facets of the recertification process may include, but need not be confined to:

1. Participation in approved CME courses.
2. Cognitive examinations focused to individual practice types.
3. A peer review process.
4. Proficiency programs for pathologists.
5. A practice assessment component.
6. Laboratory proficiency and accreditation programs.

• The recertification process should include reasonable methods to assess ethical, behavioral, alcohol and drug impairment aspects of pathologists' practice performance.

• Recertification may become an important quality assurance process, but should not presently affect licensure or hospital privileges.

• Any recertification process should be implemented on a trial basis with studies designed to assess its impact on improvement in pathology practice and patient care.

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Inaugural Address
President, American Society of Clinical Pathologists

C. Barrie Cook, M.D., M.B.A.

Doctor Lundberg, fellow members of the American Society of Clinical Pathologists (ASCP), honored guests, ASCP staff, families, and friends.

I am honored to be the President of the ASCP for the next year. The list of past presidents reads like a list of Who's Who in Pathology and I feel most humble. When I became President of The Medical Society of Virginia, I felt that I had reached the pinnacle of my professional life, as that represented acceptance and recognition by nonpathologists, which had only happened on one other occasion in Virginia, when Dick Palmer was the President of that Society. However, to be the President of the American Society of Clinical Pathologists and to be recognized by one's peers in the same discipline even surpasses that previous honor.

Last year, George Lundberg made mention of the Three Georges, I, II, and III; or, as we know them, Drs. Stevenson, Hoffman, and himself. We are proud of all three and what they have done for our Society. Our George, the III, carried out the duties of his office this past year in a superb manner and was superior to his namesake in English history, who had some big problems. In that context I would like to say that this year begins the reign of Charles I, or Charlemagne, who was an illustrious leader in his time. I hope to emulate some of his accomplishments and not to cloud his place in history by a poor performance because of name association.

How was I, or any of our previous presidents, able to reach this position of importance without the help or inspiration of others we have known or been associated with. If my godfather, Dr. William J. Mallory, an internist in Washington, had not suggested that I take "a year" of pathology to understand better the pathophysiology of disease, I doubt that I would be here. If Dr. Lee Sutton, Chairman of the Department of Pediatrics at the Medical College of Virginia, had not suggested that I go to the Mallory Institute in Boston, I would have not been stimulated by the likes of Dr. Fred Parker, G. K. Mallory, Stanley Robbins, Dante Compagne-Pinto, and others in Boston. I doubt that I would be here today. If it were not for my associates in pathology at Fairfax Hospital, Drs.

Lineberger, Hefter, Athari, Pastore, Sennesh, Hostetter, and Kankam, as well as the others in our group at American Medical Laboratories such as Drs. Godwin, Brown, Dauber, Kheir, Ocampo, and Kerman, who have picked up the slack when I have been gone, I doubt that I would be here. Also, the hospital administration at Fairfax Hospital, as well as the medical staff at the institution have tolerated my involvement in this and other organizations.

C. Barrie Cook, M.D., M.B.A.

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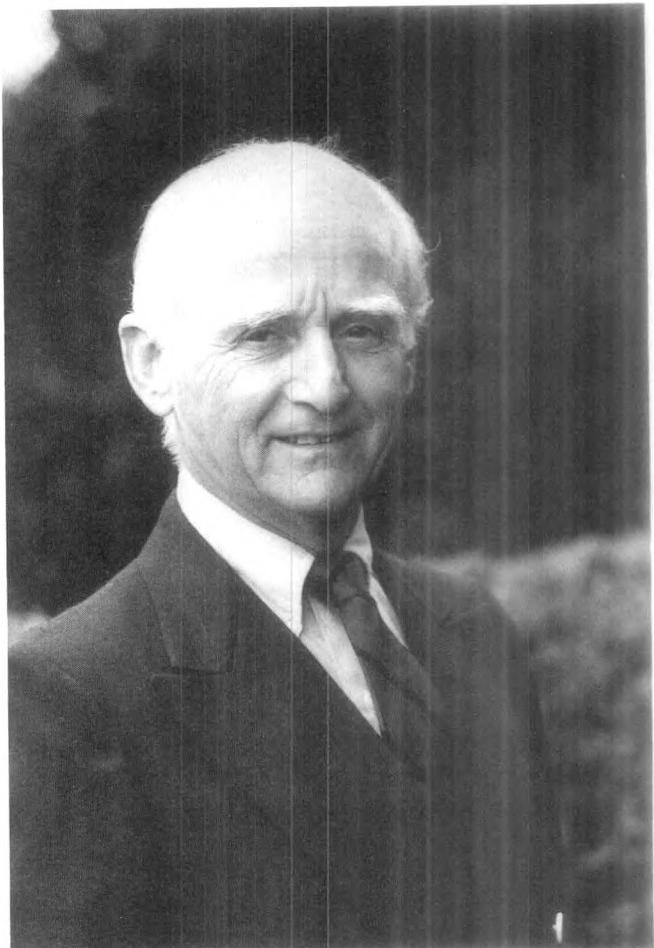
All the above were important, but if I did not have my understanding, supportive, and loving wife, Jean, and five wonderful children, who have always been helpful, I know I wouldn't be here. I cannot help but thank Dr. Frank Vellios, who had the foresight and wisdom to appoint me to serve as Assistant Treasurer under Herb Krickstein, from whom I learned a great deal (especially how to lose money on the golf course). To you, one and all, I say "thanks" and I hope your confidence will be justified, and I know your help and advice will be needed.

In my earlier years in pathology I was involved with the College of American Pathologists (CAP) as an inspector and took a number of their management courses. Benwood Hunter stimulated my interest in nuclear medicine and in blood banking. As a result, I became active in the American Association of Blood Banks and was on their I and A Committee, and at one time was the Chairman of that Committee, and for that I thank Dr. John Shiveley. In the ASCP I served on the Advisory Council for 10 years. It was there that I became aware of the many individuals who contributed their time and energy to improve the practice of pathology by extending their knowledge and expertise to others. It reminded me of an article in the Wall Street Journal. The article was about the National Endowment for the Arts and how that organization believes that quality above all is what endures in the Arts. This is what, to me, motivates those in the ASCP, both volunteers and staff. The National Endowment for the Arts has exhibited risk taking and experimented in different forms of art, some of which have been successful, others, not. This has also been true of the ASCP in their different programs and teaching innovations. It is through the courage to fail that we achieve eventual success. Because I had insufficient expertise to teach, I decided to use whatever talents I had to help make the job of the volunteer teachers easier, and the ASCP a more viable organization. Therefore, as a member of the Finance Committee and Treasurer, I was viewed as being parsimonious and penny pinching, when I really wanted to ensure the continuation of the ASCP, and to make the other members of the Board and the staff aware that we had to be solvent and accountable to our membership that had placed its trust in us. I'm happy to say that during my tenure as Treasurer our total assets increased by approximately four million dollars.

Last year my predecessor enumerated the 17 goals of the Society that were established by the Research Development and Strategic Planning (RDSP) Committee under his guidance. These goals had been prioritized by the Delphi process, which we participated in and became quite familiar with. He then listed those 9 initiatives he wished to achieve. I might say that he has done extremely well, but fortunately for me he did not complete all of those,

and therefore there are a few things that remain unfinished to provide a challenge for me.

The first is membership, which is the first priority on my list, as it was on his. This is our life's blood, so to speak. Our membership is getting older and fewer people are entering the field as either pathologists or medical technologists. New and pending government regulations do not make the field as attractive, but this is true for all of medicine. We will continue to work with the CAP, the Association of Pathology Chairman (APC), and other organizations to change this, and to prevent the impending manpower shortage in pathology, which should help our membership as well. I have asked Alma Kuby to continue her excellent work in this area as continuing effort is needed to obtain new members in all categories, especially resident members.

The next area of concern is the financial solvency of the Society. This is essential for a vigorous approach to the many new challenges facing us at this time. We need to maintain our preeminence as an educational organization. As I mentioned earlier, to maintain quality and take chances on new and innovative projects, it is essential to be a solvent society.

A third area of concern to me is communication between our Society and the other pathology organizations. As some of you know, there are many people in the American Medical Association (AMA) who think there are too many pathology organizations and too many pathologists in the AMA as delegates. Indeed, we do have a disproportionate number of delegates in that organization compared to other specialties and their comparative size. Therefore, it is important for us to communicate more clearly and more often, so that there are fewer misunderstandings and more areas of agreement. Concentration of effort in clearly defined areas is essential. Some of you may be aware that the officers of the ASCP and the CAP have been meeting periodically, as have the officers of these two organizations with the officers of the APC on a separate and on-going basis. I would hope that this year the officers of these groups might meet together instead of separately, on some occasions to coordinate more effectively their actions and activities.

The next item on my agenda is direct access pathology. This, to me, is a new opportunity for pathologists. Direct access by patients to laboratory testing is a concept whose time has arrived. A number of states already allow this, and other have limited access by certain individuals. What is self-testing except direct access? Home testing kits are being developed all the time with the latest one being for AIDS. Those patients who want laboratory data interpreted could get it from a pathologist or the pathologist could refer the patients to an appropriate physician. This may seem strange to some pathologists who have little or

no patient contact, but I am sure all of us, even those who do only anatomic pathology, have come in contact with those patients or the families of patients who wanted to speak directly to the person who looked at the slide and made the diagnosis. Finally, this could result in savings for patients and third-party payors for Special Projects.

The Past Presidents Committee for Special Projects, which has been working under the direction of Dr. Dolan, will continue, and I would hope they would be able to raise sufficient funds to enable one or two young pathologists and/or medical technologists to spend a 6- to 12-month period of time at the ASCP headquarters. Here they could work on special projects and become more familiar with the workings of the organization, which would give us a chance to scout them as well. In addition, they would bring in challenging new ideas for consideration. The young are never afraid to try something new. To accomplish this, a dedicated staff with a more organized approach is necessary.

The RDSP Committee will continue to refine the old goals and develop new ones, as indicated, which my successors will have to grapple with. An update by this Committee is appropriate so incomplete and yet-to-be-started goals or strategies will not be overlooked or lost.

Last, there are many other important areas, such as Ethics and Practice Parameters. The chairmen of these committees, Drs. John B. Henry and Rex Conn, are working diligently and we await the results of the activities and deliberations of their committees.

These are challenging and exciting times for pathology and the ASCP. We are all concerned about new government regulations and actions that have been taken. I have said nothing about these, as our area of interest and expertise is mainly in the field of education, while our co-society, the CAP, is meeting that challenge head-on, with vigor and some degree of success. Our main concern in that arena remains our associate members, whose concerns we must address. A young student contemplating a medical career was visiting our laboratory. She asked me if I would go to medical school at this time if I had to do it all over again. I thought for a moment and then allowed as how times and laws had changed, and there were a lot of problems with government becoming more intrusive in our practice. I said "I would have to think about it, but probably would, as I enjoyed what I was doing, and would go to medical school, no matter what." She thought for a few seconds and said: "Dr. Cook, I won't have those problems, as I don't know how it was."

What is preceived as adversity by some will appear as opportunity to others. As I see it, our biggest problem is communication with each other. This is, in part, because our words get mixed up. A friend told me of a sign in a small restaurant he had seen in the Sudan region of Africa. It reads: "The water in this restaurant has been personally passed by the manager." Passed is a good word. "Approved", however was the right word. Some things are good, but not right. Finding the right word, or words, is not easy, but now is the time.

Minutes of the Annual Business Meeting of the American Society of Clinical Pathologists, Tuesday, October 23, 1990

Dallas, Texas Convention Center

Presidential State of the Society Address—George D. Lundberg, M.D.

The Sixty-eighth Annual Business Meeting of the American Society of Clinical Pathologists was held in West Ballroom C of the Dallas Convention Center on Tuesday, October 23, 1990. George D. Lundberg, M.D., President, called the meeting to order at 8:30 A.M. He welcomed everyone to the meeting and appointed Mr. Altman as the official parliamentarian.

In his inaugural address, Doctor Lundberg pledged to uphold the mission statement of the ASCP to the best of his ability, and to his knowledge has done so. The Board of Directors, volunteers, staff, and a host of others have labored within the mission statement toward the 17 official goals of the Society. A report on the nine initiatives announced last year follows.

1. Membership. Many efforts have been made and continue to be made in this area. The results are more than 3,000 more members, including Physicians/Fellows, Residents, and Associate Members (Grade B+).

2. Ethics. This committee created and recommended approximately 25 principles in the fields of membership, the practice of pathology, and laboratory medicine. A survey of 1,000 Fellows assisted the Board of Directors in approving 24 ethical principles as policy (Grade A+).

3. Financial Security. Total revenue for fiscal year 1990 was 9% more than fiscal year 1989 and the total assets increased by approximately 1.3 million dollars (Grade B+).

4. Fund Raising. The Past Presidents Fund Raising Committee developed a concept for fund raising and is looking for focused projects. It has raised several thousand dollars from its own ranks (Grade B−).

5. New Practice Patterns. The ASCP Direct Access Pathology Committee recommended new policies on direct patient access that the Board of Directors adopted. Articles on this subject will soon appear in *ASCP NEWS, Laboratory Medicine, The Archives of Pathology & Laboratory*

Medicine, and the *Journal of the American Medical Association.* This policy has been transmitted in many forms to the United States government (Grade A+).

6. Practice Parameters/Guidelines. This new committee is working hard and getting off the ground slowly in a difficult area. Doctor Lundberg predicted that the future looks bright in very troublesome times (Grade B−).

7. Intersociety Relations. A Joint Officers Committee met four times during the past year. The ASCP and CAP have different purposes and different constituencies but do have much in common. A greatly improved relationship with the Association of Pathology Chairman has developed over this past year (Grade B).

8. Assuring Continuing Competence. The second ASCP Colorado Springs Conference was held in May 1990 and developed a formal policy on the subject of board recertification. Using this and other input, the CAP and the American Board of Pathology also have developed position statements (Grade A).

9. Recognition of Distinguished Service to Medicine. This award was presented last year to Doctor Koop and was a high point of the year (Grade A).

American Journal of Clinical Pathology. Mark R. Wick, M.D., has replaced retired Editor Myrton F. Beeler, M.D., for the *American Journal of Clinical Pathology.*

Doctor Lundberg acknowledged and thanked Robert A. Dietrich, M.D., J.D., the Executive Vice President, for his dedicated service to the Society and for making his year as President easier. In addition, Doctor Lundberg praised George F. Stevenson, M.D., for his untiring years of service on behalf of the ASCP.

Necrology Report—Robert W. Christie, M.D.

Doctor Christie paid tribute to the memory of the Society's recently deceased members with a reading from the Ninetieth Psalm.

The following names of the Society's departed colleagues were read:

Necrology Report
(October 1989 to October 1990)

Emeritus Fellows

Stuart M. Anderson, M.D.	Lovell, Maine
Jacob Joseph Baron, M.D.	Milwaukee, Wisconsin
Chapman H. Binford, M.D.	Arlington, Virginia
William C. Black, M.D.	Denver, Colorado
Gloria G. Brennan, M.D.	Falls Church, Virginia
Sylvia Bubis, M.D.	Columbus, Ohio
Henry Joseph Caes, M.D.	Newark, Ohio
Guillermo M. Carrera, M.D.	New Orleans, Louisiana
Albert Ehrlich, M.D.	Fort Lee, New Jersey
James W. Erchul, M.D.	Menasha, Wisconsin
Howard J. Gomes, M.D.	San Jacinto, California
James E. Habegger, M.D.	Naples, Florida
Samuel J. Hoffman, M.D.	Chicago, Illinois
Robert T. Iwata, M.D.	Roseburg, Oregon
Harold Kade, M.D.	Springville, California
Eugene W. Lange, M.D.	Lehigh Acres, Florida
Jeff Minckler, M.D.	Arcata, California
A. Ralph Monaco, M.D.	Panama City, Florida
Benjamin F. Norris, M.D.	Guilderland, New York
Avis M. Olson, M.D.	Lathrup Village, Michigan
Harold D. Palmer, M.D. (Past President 1962–1963)	Sedona, Arizona
Robert A. Peterson, M.D.	Gaylord, Michigan
Robert J. Ritterhoff, M.D.	Cincinnati, Ohio
Oscar A. Ross, M.D.	Beachwood, Ohio
Fred Ryden, M.D.	Franklin, Tennessee
Henry D. Santina, M.D.	New Orleans, Louisiana
Cameron L. Self,, M.D.	Phoenix, Arizona
Raymond B. Squires, M.D.	Cantonment, Florida
Ronald W. Steube, M.D.	Naples, Florida
Margaret C. Swanton, M.D.	Clinton, North Carolina
Henry A. Teloh, M.D.	Hillsboro Beach, Florida
Arthur H. Wells, M.D.	Duluth, Minnesota

Fellows

John Douglas Blair, M.D.	St. Lous, Missouri
John R. Buscher, M.D.	Sweickley, Pennsylvania
Stuart A. Chamblin, Jr., M.D.	San Antonio, Texas
Edward Coffey, Jr., M.D.	Manhasset, New York
John W. Colton, M.D.	Santa Monica, California
Arnold A. Effron, M.D.	Bayside, Wisconsin
Alberto G. Gerdts, M.D.	Lubbock, Texas
Eugene H. Kagan, M.D.	Syracuse, New York
Paul E. Kaldahl, M.D.	Oklahoma City, Oklahoma
Louis Ramon Reveley, M.D.	Houston, Texas
Verne A. Schulberg, M.D.	Aitkin, Minnesota

Inactive Fellow

Bruce Anders Lloyd, M.D.	Salt Lake City, Utah

Clinical Scientists

John Cornyn, D.D.S.

Margaret Edna Todd, Ph.D.

San Antonio, Texas

Englewood Cliffs, New Jersey

Foreign Fellow

Chai Hong Chung, M.D.

Taegu, South Korea

Report of the Treasurer—Richard E. Horowitz, M.D.

Doctor Horowitz presented the Society's financial statement and a revenue and expense report, which were also distributed to the audience. He further reviewed the fiscal components of the Society with regard to revenue compared to expenses for different departments within the ASCP. He further indicated that the upcoming year appears financially, educationally, and scientifically superb.

On September 14, 1990 a report was received by the Board of Directors on an independent audit conducted by the accounting firm, Deloitte and Touche, of Chicago, Illinois. The audit found no material weakness or irregularities in the balance sheet, financial statements, or in the internal financial control structures of the ASCP.

After discussion, a motion was made, seconded, and adopted unanimously that:

The calendar year 1991 dues for the following classifications of membership be adopted:

Fellow	$250
Clinical Scientist	85
Medical Affiliate	85
Foreign Fellow	100
Foreign Clinical Scientist	50
Resident	35
Associate Member	25
Active Emeritus	100

Report of the Secretary—Philip L. Barney, M.D.

Doctor Barney reported that the total ASCP membership is 47,911, with increases in all categories including Fellows, Residents, Emeritus Fellows, and Associate Members. The Board of Directors adopted several policy and position statements during the past year such as:

A policy on Direct Access Testing

A policy on Direct Billing for Clinical Laboratory Procedures that require a professional component

A Recertification Statement

A policy on Co-sponsorship Programs

A Conflict of Interest Statement which will be disseminated to the membership

An ASCP Award for Academic Excellence was presented to 79 outstanding sophomore medical students in the United States. This was an effort to provide incentives to medical students considering pathology as a career in medicine.

After discussion, Doctor Barney moved, it was seconded, and adopted unanimously that:

The minutes of the October 31, 1989 ASCP Annual Business Meeting be approved as published.

Report of the Executive Vice President—Robert A. Dietrich, M.D., J.D.

Doctor Dietrich reported that the Society continues to be even more vital today than in the past in addressing our membership's continuing educational needs, certification activities, as well as recruitment activities. A detailed report is attached to these minutes as Appendix A.

Report of the CAP President—Loyd R. Wagner, M.D.

Doctor Wagner commented on activities the CAP has considered during the past six months. The most demanding has been the preparation of responses and meetings associated with the proposed CLIA 1988 implementation regulations. The CAP prepared extensive comments on the final regulations, objecting to many of the provisions in both general laboratory requirements and particularly in cytology.

Attempts have been made to obtain relief from the Internal Revenue Service initiatives concerning affiliated service groups and leased employees. It could place a heavy administrative and fiscal burden on pathologists to assure compliance with the IRS rules. The CAP is attempting to gain a prospective application only if they are finalized and is seeking safe harbor relief.

At the September Board of Governors meeting, a report was received from the task force on recertification and referred to the House of Delegates for input before final action in November. The recommendations oppose mandatory recertification and endorses the establishment of a voluntary multiple pathway recertification methodology as proposed by the American Board of Pathology.

Report of the Commissioner of Continuing Education— Karen M. Ireland, M.D.

Doctor Ireland announced that this 1990 Fall meeting is the most successful ever in attendance, participation,

and workshops. She elaborated on specific areas of the CCE with regard to quality assurance, the CheckPath program and a new educational product called Cytotest.

A third cytology video disc is available that addresses the needs of the membership for continuing education in this area. S. Raymond Gambino, M.D., has edited the Check Sample program for 21 years and it continues to do well. This year, Doctor Wetli will assist Dr. Gambino in the anatomic pathology program as associate editor. The teleconference series also has been very successful and has been supported by a joint committee of the Associate Member Section (AMS) and CCE that develops new ideas for approximately 60 new programs each year. Last, Russell Brynes, M.D., has accepted the appointment of Deputy Commissioner of the CCE.

Report of the Commissioner of Graduate Medical Education in Pathology—M. Desmond Burke, M.D.

Doctor Burke gave an update on the activities of the CGMEP. This past year the Commission expanded from a single Commission of nine individuals to two councils. One council deals with residency training and the in-service examination and the other council handles matters concerning research and education. Anatomic pathology is tested annually, but the clinical pathology sections are on a two-year cycle. While there have been requests to test all sections of pathology each year, the CGMEP has decided not to make any changes and/or additions, but will expand the number of items in each segment of the examination. Last year's Basic Science Research Symposium and the Residency Program Directors Conference were very successful both in attendance and content.

Report of Current Board of Registry Activities—Paul J. Cherney, M.D., Chairman

Doctor Cherney addressed current activities of the Board of Registry. The primary goal of the BoR in 1990 was to develop and apply standards and procedures for certification and registration of medical laboratory personnel. A joint task force on Recruitment, Retention, and Image continues to work with the membership of the AMS. An *ad hoc* committee has been studying computer-adaptive testing and a field test was conducted last year. All examination committees are working to enlarge the pool of examination items and the feasibility of using computer-adaptive testing in the future.

The Research and Development Committee studied the examinations and the performance of various individuals coming from different eligibility routes and has confirmed once again that the highest examination grades

are earned by those individuals from CAHEA accredited training programs.

A new project of the Research and Development Committee was to investigate methods for the recognition of individuals with special qualifications in laboratory areas that are not broad enough for usual certification. The committee is analyzing a number of possible mechanisms by which such special qualifications could be recognized and has conducted some surveys to help indicate the need for this activity.

The most recently established and successful certification programs are in phlebotomy and laboratory management. Data from last year's survey of educational programs indicates an increase in cytotechnology programs and students entering this field, and a slower rate of decline of several other programs including the largest—medical technology.

Report of the Advisory Council Chairman—Anna R. Graham, M.D.

Doctor Graham reported that the Advisory Council continues to act as a liaison to bring concerns and issues from all pathologists to the Board of Directors. Discussions during the past year have addressed issues of direct access to laboratory testing, medical technologists shortages, and federal legislation impinging on laboratory practice. Through the ASCP Washington Report, councilors have been able to respond promptly to impending federal legislative and regulatory changes, including review of last year's model lab personnel licensure legislation.

At the 1989 Fall Meeting in Washington, DC, Doctor Henry Desmarais, of Health Policy Alternatives, Inc., spoke about pathology issues and the policy environment of Medicare. Dr. Wayne Smith, of the Health Care Financing Administration, discussed the evolution and impending implementations of CLIA 88 at the 1990 Spring Meeting in San Francisco.

Report of the Associate Member Section Chairman—Anne Thompson, M.T.(ASCP)S.H.

Ms. Thompson presented a brief update on the Associate Member Section. She reported that the structure of the AMS now includes an elected Administrative Board, 12 committees using more than 65 volunteers, and a network of more than 65 state volunteers acting as advisors. During the past year, the AMS held five regional workshops with an attendance filling more than 4,000 spaces. All the volunteers have worked to make this AMS year the most successful. Ms. Thompson introduced the 1991 Chairman of the AMS, Ms. Polly Cathcart, M.M.Sc., M.T.(ASCP)S.C.

Report of the Resident Physician Section (RPS) Chairman—Daniel L. Hood, M.D.

Doctor Hood reported that during the past year the number of Resident physician members has increased by 15%, for a total of 1,294 members. This represents the largest increase of any member category within the Society. During the past year the RPS presented two resident-oriented programs at the national meetings. The 1990 spring meeting program focused on types of practice environments residents will face when leaving training programs. At this 1990 Fall meeting, the program was on publishing medical papers by resident physicians. Currently members of the Administrative Board of the RPS participate on the Board of Directors, the CCE, the Membership Committee, and the Communications Committee. The 1990–1991 Chairman of the RPS is Timothy C. Greiner, M.D.

Report of the Government Relations Committee—Rex B. Conn, M.D., Chairman

Doctor Conn reported that the major activity of the Government Relations Committees was reviewing the many documents, including the federal register related to CLIA 1988, commenting on the various components on this legislation/regulation, and drafting recommendations to HCFA from ASCP. Doctor Conn noted several different activities in the CLIA 1988 regulations that the ASCP should support or oppose.

The Government Relations Committee also drafted responses opposing home blood collection for human immunodeficiency virus testing and supporting direct access to laboratory services by patients. Doctor Conn noted that the Committee and the Washington Office function as resources to provide information and to advise, whenever possible, on any type of legislation or regulations related to the laboratory.

Practice Parameters Committee Status—Rex B. Conn, M.D., Chairman

Doctor Conn gave the definition of a practice parameter as an acceptable strategy for diagnosis or treatment of a specific clinical condition. He commented that there is no question that the concept of practice parameters is here to stay. Approximately 40 organizations and governmental agencies are drafting practice parameters. The federal government is providing financial support and has assigned responsibility for drafting practice parameters to certain federal agencies. The AMA is functioning as a central clearing house for information and will continue to be involved.

Writing a practice parameter is simply the first step in improving medical care. Parameters must be validated,

communicated, and instituted. Using a standardized protocol and format developed by the Committee for evaluating written practice parameters, practice parameters are being reviewed. A data base is being developed for all published practice parameters to identify and select those related to pathology or laboratory medicine. It is important now to explore the concept of validation and the protocol for validating a developed practice parameter. Last, the committee is writing practice parameters related to replacement of obsolete tests and availability of stat service while setting the ground work for an ongoing critical approach to the whole concept.

American Board of Pathology—Murray R. Abell, M.D., Ph.D.

Doctor Abell provided copies of his report and noted that one item from the report was a position statement with regard to recertification prepared early this year. The American Board of Pathology's (ABP) position statement and that of the ASCP are quite similar. Some general points from that statement include the following:

1. The ABP is the only body recognized by the American Board of Medical Specialities to certify physicians in pathology and is the only body that can recertify those physicians.
2. The ABP has a responsibility to all diplomates who desire recertification.
3. The process of recertification must be educational and meaningful.
4. Recertification in an area of pathology should be available only to those who hold a valid certificate issued by the ABP.
5. Continuing medical education and practice assessment should be major components of any recertification process.
6. The ABP should not be involved in recredentialing noncertified pathologists.

Policy positions of the ABP are listed below.

1. The ABP opposes time-limited certification and mandatory recertification.
2. The ABP approves the concept of voluntary recertification as a pathway for recredentialing.
3. The ABP approves some combination of continuing medical education, assessment of practice performance, cognizant examinations, as well as other approaches that may be developed.

Doctor Abell commented that the ABP would give its first examination in pediatric pathology on November 20, 1990. A cytopathology examination has been instituted as an area of added qualification, with 475 individuals certified in this area.

Presentation of Awards—George D. Lundberg, M.D.

Doctor Lundberg recognized all of the 1990 ASCP award winners.

Philip Levine Award:	Walter T. J. Morgan, Ph.D., D.Sc., and Winifred M. Watkins, Ph.D., D.Sc., F.R.C.
H. P. Smith Award:	William M. Christopherson, M.D.
Ward Burdick Award:	S. Raymond Gambino, M.D.
Jt. Distinguished Service Award:	Pierre W. Keitges, M.D.
Israel Davidsohn Award:	Joseph C. Sherrick, M.D.
Technologist of the Year:	Ann H. Clark, M.A.T., C.T.(ASCP)
Sheard Sanford Award:	Patricia M. Kopko and Timothy C. Cripe, M.D., Ph.D.
CCE Distinguished Service Award:	David H. Dail, M.D. Robert E. Fechner, M.D. Karen M. Ireland, M.D. Janice M. Nelson, M.D. Paul P. Sher, M.D.
AMS Distinguished Service Award:	Barbara J. Minard, M.T.(ASCP)S.H., S.I.

Report of the Bylaws Committee—Karn M. Ireland, M.D.

Doctor Ireland reported that the Bylaws Committee is proposing several changes to the ASCP bylaws. After discussion a motion was made, seconded, and adopted unanimously that:

The proposed amendments to the ASCP bylaws be approved as circulated. (Appendix B).

Report of the Nominating Committee—George C. Hoffman, M.D.

Doctor Hoffman presented the Nominating Committee report for the 1990–1991 year. The following nominations for the Society's officer positions were presented:

President-Elect:	Ernest S. Tucker III, M.D. San Francisco, California
Vice President:	Thomas A. Bonfiglio, M.D. Rochester, New York

The motion was made, seconded and adopted unanimously that:

The nominations be closed and unanimous ballot be cast for the slate.

Doctor Hoffman presented the following nominees for the At-Large Members of the Board of Directors: J. Scott Abercrombie, Jr., M.D., Boston, Massachusetts; Stebbins B. Chandor, M.D., Huntington, West Virginia; Ralph A. Korpman, M.D., San Bernardino, California; and Janice M. Nelson, M.D., Los Angeles, California.

The motion was made, seconded and adopted unanimously that:

The nominations be closed and unanimous ballot be cast for the slate.

Doctor Hoffman presented the following nominations for the Commission on Continuing Education and the Commission on Graduate Medical Education in Pathology.

Commission on Continuing Education

Council on Anatomic Pathology	Robert E. Fechner, M.D. Charlottesville, Virginia
	Arthur F. Patchefsky, M.D. Philadelphia, Pennsylvania
	Robert E. Petras, M.D. Cleveland, Ohio
	Robert E. Scully, M.D. Boston, Massachusetts
	Fattaneh A. Tavassoli, M.D. Washington, DC
Council on Forensic Pathology	Marcella Fierro, M.D. Richmond, Virginia
	Kris L. Sperry, M.D. Atlanta, Georgia
	Larry R. Tate, M.D. Columbus, Ohio
Council on Cytopathology	Michael D. Glant, M.D. Indianapolis, Indiana
	Geraldine O. Horak, S.C.T.(ASCP) Cleveland, Ohio
	Thomas F. Kardos, M.D. San Antonio, Texas
Council on Hematology	John B. Cousar, M.D. Nashville, Tennessee
	LoAnn C. Peterson, M.D. Minneapolis, Minnesota
	John T. Brandt, M.D. Columbus, Ohio
Council on Transfusion Medicine	Benjamin Lichtiger, M.D., Ph.D. Houston, Texas
	Joel M. Umlas, M.D. Cambridge, Massachusetts
	Margaret E. Wallace, M.T.(ASCP)S.B B. Shreveport, Louisiana
	Rosalyn A. Yomtovian, M.D. Cleveland, Ohio
Council on Clinical Chemistry	George C. Cembrowski, M.D. Minneapolis, Minnesota
	Jay L. Bock, M.D. Stony Brook, New York
	Richard A. McPherson, M.D. San Diego, California
Council on Immunopathology	James A. Goeken, M.D. Iowa City, Iowa
	Roger S. Riley, M.D. Greenville, North Carolina
	John A. Carey, M.D. Detroit, Michigan
	Barbara J. Minard, M.T.(ASCP)S.H., S.I. Wichita, Kansas
Council on Microbiology	Paul Valenstein, M.D. Ann Arbor, Michigan
	Tsieh Sun, M.D. Manhasset, New York
	Ron B. Schifman, M.D. Tucson, Arizona
Council on Special Topics	Diane C. Farhi, M.D. Cleveland, Ohio

Lucia B. Berte, M.S.,
  M.T.(ASCP)S.B.B.
Elmhurst, Illinois

Lee H. Hilborne, M.D.
Los Angeles, California

Council on Medical Informatics   Donald Connelly, M.D.
                                     Minneapolis, Minnesota

                                     Evan M. Cadoff, M.D.
                                     East Brunswick, New Jersey

Commission on Graduate Medical Education in Pathology

Council on Education &   Peter J. Goldblatt, M.D.
Research   Toledo, Ohio

                                     Robert L. Treldstad, M.D.
                                     Piscataway, New Jersey

                                     Jay M. McDonald, M.D.
                                     St. Louis, Missouri

Council on Resident Training   Steven G. Silverberg, M.D.
                                     Washington, DC

                                     Mark R. Wick, M.D.
                                     St. Louis, Missouri

The motion was made, seconded, and adopted unanimously that:

The nominations be closed and unanimous ballot be cast for the slate.

Induction of Newly Elected Officers and Board of Directors—George D. Lundberg, M.D.

Doctor Lundberg presented Doctor Cook with the gavel and President's badge, noting his acceptance of the Office of the President for the coming year. Doctor Lundberg then presented the newly elected officers and members of the Board of Directors with their badges: Ernest S. Tucker III, M.D., President-Elect; Thomas A. Bonfiglio, M.D., Vice President; reelected Board Members, J. Scott Abercrombie, Jr., M.D., Ralph A. Korpman, M.D., Janice M. Nelson, M.D., and new Board member Stebbins B. Chandor, M.D. Doctor Cook's first duty as Incoming President was to present Doctor Lundberg with a plaque and pin from the Society together with his Past President's badge in recognition of his outstanding leadership during the past year.

Inaugural Address of the President—C. Barrie Cook, M.D., M.B.A.

Doctor Cook gave his Inaugural Address, the entire text of which is attached as Appendix C of these minutes.

Adjournment

There being no further business, the meeting was adjourned at 10:15 A.M.

Annual Report of the Executive Vice President

The Society continues to be even more vital today than in the past in addressing our membership's continuing educational needs, certification activities, as well as recruitment activities.

The fall national meeting attendance has increased for the past four years. The Washington Fall national meeting had a record of 9,061 registrants while its AP Slide Seminar on Diseases of the Gastrointestinal Tract had 1,170 registrants. The San Francisco Spring meeting had 6,343 registrants while its AP Slide Seminar on Diseases of the Thyroid had 906 registrants.

During 1990, two Weekends in Pathology, in Orlando and New York were presented; 61 teleconference programs to more than 4,700 sites with an estimated attendance of 40,000 participants were conducted; technologist workshop weeks were held in Anaheim, Chicago, Boston, and Williamsburg under the aegis of the Associate Member Section; 25 in-depth educational courses were presented in a variety of locations nationwide and the Pathology Update Course was presented this year in Charlottesville, Virginia.

In May of this year, the Colorado Springs Conference was held under the leadership of Doctors McKenna, Smith, and Lundberg. This was a landmark national conference on the general subject of recertification and physician competence that resulted in a major ASCP position paper on recertification, previously published. The video disc project successfully field tested its first product, *CytoScreen I* and completed *CytoScreen II*. Both of these video discs are designed to simulate screening and diagnosis of Pap smears of the female genital tract. A third cytopathology disc based on the *Check Sample* series was also prepared.

ASCP Press and Subscription Activities continues to enhance the number of books and other materials available. During the past year, 13 new titles were published, among which are *Laboratory Quality Management* by Cembrowski; *Nucleic Acid Probes* by Piper; a revised edition of the *ABC's of LIS* by Elevitch; *Developing Performance Standards* by Berte; and an *SI Unit Pocket Guide* by McQueen. In addition, three colored texts, *Pathology of the Mediastinum* by Flinner; *FNA II:Lymph Node, Thyroid, and Salivary Gland* by Feldman; and *Intraoperative Consultation* by Nochomovitz were published. New video tapes available include *Blood Collection: The Pediatric Patient* by Phelan; *Laboratory Safety and Infection Control* by Luebbert; and *Pap Smear: Collection, Handling and Quality Assurance* by Somrak.

Just released or soon to be are *Breast Pathology* by Fechner and Mills; *Testicular Tumors* by Young and Scully; *Clues to Diagnosis in Dermatopathology* by Ackerman; *Cytopathology of the Uterus and Cervix* by Meisels; *Histotechnology: A Self Instructional Text* by Carson; and *Cutaneous Adnexal Tumors* by Wick.

Cytology has taken a high profile during the past year, which resulted in the establishment of a Cytopathology Education Consortium by the Board of Directors. A membership need for reference materials in cytopathology is being addressed by the

recently published *Cytotest/A Practice Exam in Cytopathology* and the *Pap Smears* video tape by Somrak. Other cytopathology titles forthcoming will include *Cytostack,* a hypercard cytology text book, as well as a product based on the collected text of the cytopathology *Check Samples.* In production are two new video tapes, one in histotechnology and the other on nonroutine blood collection techniques. Other new products include *Computer Adapted Instruction Software on Blood Collection Techniques* and a computer adapted testing product to complement an all new edition of the *Board of Registry Study Guide.* Other forthcoming titles include *Implementing QA* by Bozzo, *Transfusion Transmitted Diseases* by Smith; the *Customer Orientated Laboratory* by Umiker; and *Immunohistopathology* by Elias.

During the past year, *Laboratory Medicine* as well as *ASCP NEWS* completed the transition to desk top publishing. Since the May 1990 issue of *Laboratory Medicine,* all composition work has been produced in house, resulting in a significant reduction of costs. During the summer, the *American Journal of Clinical Pathology's* editorial support function was relocated to the ASCP headquarters with on-line computer access established between the editor's office in St. Louis, the ASCP, and our Philadelphia-based publisher.

Check Sample and *Tech Sample* continue to prosper. Significant increases have been seen in the new QA program, *CheckPath.* Introduced in 1989, *CheckPath* has continued to increase in size with offerings in anatomic, cyto, and hematopathology. A new quality assurance program offered at national meetings is the *Challenge Examinations.* These examinations are available in anatomic, cyto, and hematopathology. A new examination in anatomic pathology is also being introduced at this meeting and a new cytopathology examination will be offered at the Spring 1991 in Nashville. These examinations provide immediate feedback and discussion of diagnoses in the workshop setting.

In response to requests from both residents and program directors for a more comprehensive test, plans are under way to expand the annual resident in-service examination. This past year a record high of 2,500 residents took the examination.

More than 2 million mailings of attractively designed catalogs and brochures were sent to our membership and the general medical laboratory community informing them of our educational offerings. More than 30,000 orders and registrations were processed by telephone. Significant efforts have been made during the past year to enhance public knowledge regarding pathology and laboratory medicine. These public information efforts are under the guidance of the Communications Committee chaired by Doctor David Smith. These efforts have resulted in public education brochures, press releases, and media kits for our national meetings. Our press releases have generated more than 750 articles in newspapers, with readerships totalling 4 million as well as in several magazines, including *Modern Maturity* and *Readers Digest,* with a combined circulation of 38 million people.

Total membership is now more than 48,000, reaffirming our position as the largest organization of pathologists and medical laboratory personnel in the world. The career recruitment network continues to grow and now includes 1,400 laboratory re-

source persons and 2,000 high school science teachers. This network is supported by staff-developed materials as well as surveys that document current personnel shortages and salaries.

A "Member Get a Member" campaign is in progress, with complimentary trips to various cities, including one to Europe and one to Hawaii, as well as weekends in various cities and cash prizes.

The Board of Registry for 1990 has more than 145,700 registrants, the largest in its history. The number of applicants for certification in 1990 was 13,298, which is approximately the same as in 1989.

The internal functions of the Society continue to be reexamined and updated for better efficiency and service to our members. The staff looks forward to another productive year.

APPENDIX B

1990 Proposed Amendments to the ASCP Bylaws

The Annual Business Meeting of the Membership of the American Society of Clinical Pathologists was held on Tuesday, October 23, 1990 at 8:30 A.M. in the West Ballroom C of the Dallas Convention Center. ASCP Fellows were asked to vote on the following proposed amendments to the Society's Bylaws during the October 23, 1990 meeting.

Philip L. Barney, M.D
Secretary

Article VI. Officers and Board of Directors.

SECTION 2. Board of Directors. The Board of Directors shall be composed of the Officers, the Immediate Past President, six At-Large Directors, the Chairman of the Advisory Council ex officio, the Chairman of the Associate Member Section ex officio, the Chairman of the Resident Physician Section ex officio, the Commissioner of Continuing Education ex officio, the Commissioner of Graduate Medical Education in Pathology ex officio and the Executive Vice President ex officio without vote. Each At-Large Director shall serve for a term of three years and may serve for two full terms or until a successor is qualified. In the event that any At-Large Director is elected as a Commissioner or to any other position whereby that person becomes an ex officio member of the Board of Directors, that At-Large Director position shall become vacant and shall be filled for the unexpired term by the President with the consent of the Board of Directors election in accordance with Article 9 of these bylaws.

Article IX. Vacancies.

SECTION 2. Anticipated Vacancies. When a vacancy in an elected office is anticipated to occur at the time of the next annual business meeting, the nominating committee shall be responsible

for nominating a candidate for election to the anticipated vacant position.

SECTION 3. Other Vacancies. Vacancies occurring by res-ignation, death, incapacity or removal in any other office, or in any other elective or appointive position shall be filled by the President with the consent of the Board of Directors. The person so appointed shall serve until the conclusion of the next annual business meeting.

Article X. Committees.
SECTION 7. Educational Materials Advisory Committee.
(b) Composition. The Educational Materials Advisory Com-mittee shall be composed of six members appointed by the Pres-ident with the approval of the Board of Directors for terms of three years, the terms to be arranged so that two expire each year. There shall be no limit to the number of terms a member may serve. Appointments to this Committee shall broadly rep-resent the educational activities of the Society. With the approval of the Board of Directors, the President, upon recommendation of the Chairman, may appoint scientific advisory boards.

Wigglesworth JS, Singer DB, eds. *Textbook of Fetal and Perinatal Pathology.* Two volumes. Boston: Blackwell Scientific Publications. 1991. $250.00.

This new two-volume textbook is a meridian work in fetal and perinatal pathology. It represents a transatlantic collaboration of two editors with 31 contributors, many of whom have published extensively in the field of fetal, perinatal, and pediatric pathology, and some of whom have written their own texts. It is appropriate not only as a "comprehensive text to fulfill the needs of perinatal and pediatric pathologists for a relatively detailed reference work" but also as a reference for residents, general and surgical pathologists, and clinicians involved in the care of the fetus and the neonate.

Volume 1 covers principles and major problems in fetal and perinatal pathology, with introductory chapters on the role of pathology in modern perinatal medicine, fetal growth and maturation, classification and causes of fetal and perinatal death, and the perinatal autopsy. Other chapters give more detailed information about the pathology of abortion, the placenta, multiple pregnancy, fetal death, intrapartum and early neonatal death, malformations, chromosome abnormalities, malformation syndromes, excess fluid collection in the fetus, and congenital tumors. Chapters 4 to 10 should be required reading for anatomic pathology residents and fellows and are very useful as a reference for more experienced pathologists.

Volume 2 discusses the pathology of organ systems in the fetal and perinatal periods with emphasis on the respiratory tract, cardiovascular system, central nervous system, orofacial region, eye, alimentary tract, liver, pancreas, adrenal glands, pituitary, thyroid, parathyroid, urogenital system, musculoskeletal system, skin, and hematopoietic system. The major diseases of these organ systems are presented, discussed, and illustrated. In some cases, immunohistochemical, cytogenetic, flow cytometric, and molecular biological applications also are incorporated into the text.

All of the chapters are well written, well illustrated, and fully referenced. However, some have more recent complications of references than others, with citations from 1988 and 1989 included. Extensive tables, graphically elegant line drawings, and flow diagrams are found throughout the two volumes. The quality of the gross photographs and photomicrographs is excellent and illustrative of the key morphologic features. Parenthetically, the same photograph appears in two separate places in the text to illustrate two different aspects of congenital cytomegalovirus infection: figure 17.16, page 545, depicts microcephaly and figure 33.22, page 1273, emphasizes the blueberry muffin rash.

My succinct and enthusiastic recommendation is to buy this book. I highly recommend it. The contributions made by the 31 authors and the extensive work of the two editors have resulted in a book with a broad compass and oceanic sweep. It is a welcome and indispensible addition to the perinatal and pediatric pathology library.

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Menendez-Botet, CJ. *Hazardous Waste: Facts and Fallacies.* Washington, DC: AACC Press. 1990. $20.00.

This 44-page booklet on biomedical hazards summarizes material presented at an American Association for Clinical Chemistry workshop. The educational content and value of these summaries vary, but generally they provide useful and practical information. The first two chapters, on carcinogenic chemicals and management of radioactive wastes, as well as the last chapter on flammable and combustibles liquids, provide very basic information and each has minimal numbers of references, mainly to textbooks. The chapter on information sources for hazardous waste gives specific information of practical importance. In addition, the discussion of the types and use of various information resources is relevant and complete. Chapters 4 and 5 on management of infectious and hazardous wastes contain information that is immediately applicable to the laboratory, including specific actions that should be incorporated into safety programs. Chapter 6, entitled "Chronically Toxic Chemicals," provides a good introductory survey of the topic with mention of typical problems associated with toxic chemicals as well as specific problem with various classes of toxic compounds. The tables of toxic compounds are helpful and some of the information could be incorporated easily into a laboratory chemical hygiene plan. Advisory information on the personal protective measures was brief but pragmatic. This booklet is appropriate for safety officers, safety committee members, and medical technologists because the material offered might serve as an initial stimulus for study and lead to practical application in laboratory safety programs.

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CORRESPONDENCE AND CORRECTIONS

Plastic Embedding for Immunohistology

To the Editor:—In the recent article, "A Novel Method for Optimum Biopsy Specimen Preservation for Histochemical and Immunohistochemical Analysis," Drs. Murray and Ewen[1] describe a method of plastic embedding of tissues for histochemistry and immunohistochemistry. The authors use the technique called "freeze substitution" rather than fixation as the first step in tissue processing. Freeze substitution refers to the immersion of frozen tissue in subzero acetone to extract ice. The tissue is then infiltrated with plastic monomer and polymerized into a plastic tissue block. The authors propose that this method differs from previously described methods of plastic tissue embedding[2-5] in that those techniques employed some form of tissue fixation that potentially alters antigenic reactivity. We note that although aldehydes were the primary fixatives in most of those cited methods,[2,4,5] the method described by our group[3] employed fixation of tissue in acetone at −20 °C. One would think that the term "fixation" may be debatable in this context and that this tissue processing step also may qualify as freeze substitution. The authors' method differs from our method in only two ways: (1) we do not freeze tissue before immersion in subzero acetone, and (2) we infiltrate tissue with plastic monomer at 4 °C rather than in subzero conditions. In this sense, it would appear that the method proposed by Drs. Murray and Ewen is not actually a novel method but at best a modification of our previously published technique.[3]

Does this modification result in any advantages with regards to antigenic preservation? If it does not, the added step of freezing tissue before immersion in subzero acetone is an unnecessary step. With regard to lymphoid antigens, the authors' modifications apparently offer no advantage because similar antigens are detected (CD3, CD5, CD19, CD22, and immunoglobulin). In fact, by our method, T-cell subsets also are detectable with CD4 and CD8. The reactivities for enzyme histochemical stains also appear similar for both modifications.

Whereas we have differed with the authors in the use of the terminology to describe immersion of tissue in subzero acetone (fixation *versus* freeze substitution), it would appear that this difference in terminology is minor because the techniques and the results obtained are essentially the same. In communications with other laboratories using our methods and in subsequent papers,[6] we have called the fixation of tissue in subzero acetone a "poor man's" form of freeze drying tissue with minimum alteration of antigens.

Finally, those of us who, over the years, have advocated plastic embedding for diagnostic immunohistology must realize that this advocacy is an uphill struggle. Plastic embedding techniques such as those cited will always have a rightful place in investigative work, but it is unlikely that they will be employed universally for diagnostic purposes. The future of diagnostic immunohistology is in more sensitive and specific reagents for the detection of differentiation antigens in routinely processed tissues, rather than in fundamental changes in the ways we process tissues. This reality becomes more apparent each year with the advent of markers such as paraffin-reactive antibodies to CD3[7] and CD20,[8] and of other antibodies to antigens previously undetectable in paraffin. Thus, in the evolutionary tree of immunohistology, plastic embedding is not part of the trunk but rather is only a branch.

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The Authors' Reply

To the Editor:—Professor Casey's letter raises several points regarding our recent paper that must be answered. First, "freeze substitution" is a process that is fundamentally different from that of fixation, even using acetone at −20 °C as the fixative, as described by Casey's group.[1] We disagree completely that the difference between our method[2] and Professor Casey's method is one of minor terminology.

The effects of tissue fixation depend on the type of fixative and the time and temperature of fixation.[3] Tissue fixation will result in a variable amount of denaturation of tissue components, and during the process of fixation there will be diffusion and extraction of soluble tissue components (enzymes, glycogen, muco-

substances, and nucleic acids, for example). In addition, degradation of tissue components during the fixation procedure also may occur (e.g., tissue nucleases may continue to digest RNA and DNA and these processes continue to operate at −20 °C). Placing unfixed, fresh nonlymphoid tissue directly into acetone at subzero temperature is likely to result in considerable ice-crystal artefact due to slow freezing of tissue water; in our experience this is particularly marked in solid tissue, e.g., of the liver and kidney. There appears to be a shell of well-fixed tissue surrounding a central core into which acetone can only penetrate slowly and this central portion of tissue is poorly preserved, with considerable morphologic disruption.

Freeze substitution, which combines rapid (snap) freezing of tissue with removal of tissue water by an organic solvent at low temperature, is a method of preserving tissue that is completely separate from fixation.[3] Rapid freezing ensures that cellular processes are stopped almost instantaneously so that degradation of tissue components does not occur. Furthermore, because the tissue is frozen, diffusion or extraction of soluble tissue components, particularly tissue antigens, cannot occur. Thus freeze substitution is a method of tissue preservation that circumvents tissue fixation and produces excellent morphologic, histochemical, and immunohistochemical results that are obtainable with a wide range of tissues, as we have clearly shown.

We have demonstrated that the activities of a wide range of enzymes, including several fixation-sensitive oxidoreductase enzymes, are maintained in freeze-substituted plastic-embedded tissue and there is accurate localization of enzyme activity with no diffusion.[4] In contrast, Casey's group only demonstrated the preservation of activity of several fixation-resistant enzymes (peroxidase, acid phosphatase, α-naphthol esterase for example); therefore the reactivities of the two methods for enzyme histochemistry are very different.

Professor Casey's comments regarding the development and use of antibodies that recognize epitopes in formalin-fixed, wax-embedded tissue appear to contradict his previous statements.[5] Formalin fixation and wax embedding is a very destructive process and the development of specific reagents to recognize antigens in formalin-fixed, wax-embedded tissue is based on the assumption that the only reason that specific antigens cannot be currently identified in wax sections is that epitopes recognized by available antibodies are denatured (destroyed) during tissue processing.[6] However, it is very likely that many antigens are extracted from tissue specimens (rather than denatured) during routine processing. Furthermore, formalin-fixed, wax-embedded sections are not suitable for enzyme histochemistry and are far from ideal for immunocytochemistry, in situ hybridization, lectin histochemistry, and the study of mucosubstances. Thus we should develop and

use novel methods to preserve tissue that ensure minimum damage or alteration to biopsies to maximize the amount of diagnostic information.[7]

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Plasmacytoid Acute T-Cell Lymphoma/Leukemia

To the Editor:—We read with interest the article by Caldwell and associates[1] regarding a case of acute leukemia/lymphoma of plasmacytoid T-cell type. Unlike all of the five[2-6] previously reported cases of plasmacytoid T-cell lymphoma, the tumor cells in this case were mitotically more active and showed more evidence of T-lineage differentiation as demonstrated by positive, although variable, staining with CD2, CD3, CD4, CD7, and UCHL-1. There was also evidence of T-cell receptor (TCR) β-chain gene rearrangement but we would note that Figure 5 was mislabeled and the extra band in lane 2 is very close to the 8.5-Kb band

sometimes seen in normal individuals. The myelomonocytic markers CD13, CD14, and CD33 were reported negative. In addition, it is the only case that had a diffuse marrow infiltration with a leukemic phase and lymphoma-related death. All preceding cases differed in that the more specific T-cell marker CD3 was consistently negative and the TCR β-chain genes of the tumor cells were in germline configuration in the case studied by Koo and co-workers.[6] The T-cell markers that were expressed (CD4 and CD5) were not lineage specific, and several myelomonocytic markers, including CD14, CD36, and CD68, often were present. All patients

died of the associated myeloproliferative disorder rather than of the nodal lymphomatous infiltrate. Indeed, the marrow was either minimally involved or uninvolved by lymphoma in all five cases.

We had the opportunity to examine two additional cases of plasmacytoid T-cell lymphoma (manuscript submitted for publication) and our findings strongly support the concept[5,6] that "plasmacytoid T cells" are of macrophage/monocyte origin as evidenced by negative βF1, CD3, and CD8 immunostaining; absent immunoglobulin heavy-chain genes; and TCR β, γ, δ-chain gene rearrangement; as well as positive CD14 and CD68. Similar

to previously reported cases, the bone marrow was involved minimally in one and uninvolved in the other. Both cases were associated with a myeloproliferative disorder that progressed rapidly to an acute myeloid leukemia.

To avoid further confusion awaiting an irrefutable confirmation of the monocytic lineage of plasmacytoid T cells, we support the use of the term "plasmacytoid T-zone lymphoma" previously advanced by Beiske and co-workers[4] for this entity. The case of Caldwell and associates[1] may be the only case that truly deserves the terminology "plasmacytoid T-cell lymphoma," which appears to be distinctive in terms of immunophenotype and clinical features when compared to plasmacytoid T-zone lymphoma.

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The Authors' Reply

To the Editor:—In reference to our article, "Acute Leukemia/Lymphoma of Plasmacytoid T-cell Type",[1] Drs. Baddoura and Chan appropriately highlight the differences in clinical presentation, morphology, immunophenotypic markers, and evolution of the disease process in the patient reported by us and those reported by others, as referenced above. Certainly, the other cases all have demonstrated evidence of myelomonocytic differentiation. In contrast, our case was solely of T-cell lineage, as evidenced by immunologic and molecular biological probes. We acknowledge the unfortunate mislabeling error in the legend of Figure 5. However, the size of the rearranged band in lane 2 of the same figure is closer to 10 Kb than the size of 8.5 Kb suggested by Drs. Baddoura and Chan. Furthermore, the presence of a rearrangement using a different restriction enzyme, BamHI, argues in favor of true clonal rearrangement, and therefore, true T-cell clonal proliferation rather than a normal germline variation due to polymorphism.

We appreciate the comments of Drs. Baddoura and Chan. Until the lineage is unequivocally determined in cases displaying myelomonocytic markers, we agree with their contention that such cases might better be described as "plasmacytoid T-zone lymphomas" to separate them from true T-cell malignancies.

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Drug-Induced Immune Hemolytic Anemia

To the Editor:—I read with interest the report by Chambers and co-workers[1] regarding possible drug-induced hemolytic anemia due to the administration of the third-generation cephalosporin, ceftazidime. However, it was unclear to me whether the *in vitro* serologic results were obtained using one of three pharmaceutical preparations available or using a chemically pure form of the drug. If only the pharmaceutical preparation was used in the serologic investigation then the conclusion of ceftazidime-induced hemolysis may not be unequivocal.

It is known that drugs supplied by pharmaceutical companies contain any number of additives or fillers[2] and that these excipient compounds could, themselves, result in misinterpretation of an investigation of drug-induced immune hemolytic anemia.[3,4] Furthermore, most excipient compounds will not be listed by the manufacturer. I raise this point to re-emphasize[4] the need for investigators to rule out any other potential causative agents contained in a given pharmaceutical drug preparation. From a scientific point of view, this is absolutely critical before we should implicate a specific drug contained in a pharmaceutical preparation. Furthermore, it is easy to obtain the chemically pure form of the drug, most of which are available from Sigma Chemical Company, St. Louis, Missouri.[4]

Obviously from a clinical point of view it is crucial to use the actual pharmaceu-

tical preparation that the patient is receiving in any initial investigation of drug-induced hemolytic anemia. Because excipient compounds are used extensively by pharmaceutical companies and cases of drug-induced immune hemolysis are rare, it may seem unlikely that any other compound contained in the pharmaceutical could be responsible. Nonetheless, *in vitro* proof of a specific drug involvement can only be provided using a chemically pure form of the suspect drug or, if available, its metabolite(s).

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The Authors' Reply

The serologic results were obtained using the pharmaceutical preparation Fortaz (Glaxo Pharmaceuticals, Research Triangle Park, NC). The preparation for injection is a dry, powdered mixture of ceftazidime and sodium carbonate, added to facilitate dissolution. Because the pharmacologic preparation in this case is essentially pure ceftazidime, we are confident that the active ingredient mediated the red cell sensitization and consequent hemolysis. Dr. Branch does well to remind us that additives should also be considered when investigating cases of drug-induced hemolysis and that confirmatory testing using chemically pure forms of implicated drugs may be required.

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Pneumocystis Ocular Infection

To the Editor:—I read with interest the recent report by Dembinski and coworkers concerning disseminated *Pneumocystis carinii* infection associated with aerosolized pentamidine.[1] They reported widespread histologic evidence of such infection after death in a patient with acquired immune deficiency syndrome.

Were the eyes examined clinically and/or at autopsy, and if so, were they involved? The authors cite the report of Kwok and co-workers[2] of retinal cotton–wool spots in a patient with *P. carinii* but apparently were unaware of some other recent reports in the ophthalmic literature.

The report by Kwok and co-workers[2] was subsequently criticized by others as failing to demonstrate that the *P. carinii* organisms caused the cotton–wool spots.[3,4] Subsequently, it has become clear that *P. carinii* typically causes a choroidal infection, clinically manifest by irregular yellow–white lesions deep to the retinal vessels.[5-7] These three papers[2,3,6] describe a total of six patients who developed disseminated infection after aerosolized pentamidine therapy, and one additional patient who had not received this drug.[6] On histologic examination, organisms were found within collections of dense amorphous eosinophilic material in the choroid. Generally, of course, this is demonstrated with autopsy examination, but one patient underwent transscleral choroidal biopsy to establish the diagnosis.[6]

The lesson for clinicians is that the ophthalmic manifestations of *P. carinii* infection may be the first evidence of extrapulmonary disease and that they may be asymptomatic.[8] Pathologists should be aware that in select cases, transscleral biopsy is helpful in establishing the diagnosis and that postmortem examination of the eyes also may demonstrate the infection.

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St. Louis, Missouri

REFERENCES

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2. Kwok S, O'Donnell JJ, Wood IS. Retinal cotton-wool spots in a patient with *Pneumocystis carinii* infection. N Engl J Med 1982;307:184-185.

3. Holland GN, Gottlieb MS, Foos RY. Retinal cotton-wool patches in acquired immunodeficiency syndrome. N Engl J Med 1982;307:1704.

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5. Dugel PU, Rao NA, Forster DJ, et al. *Pneumocystis carinii* choroiditis after long-term aerosolized pentamidine therapy. Am J Ophthalmol 1990;110:113-117.

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8. Teich SA, Rosenblatt M, Friedman A. *Pneumocystis carinii* choroiditis after long-term aerosolized pentamidine therapy. Am J Ophthalmol 1991;111:118.

Mycobacteria on Wright's-Stained Smears

To the Editor:—Having read with interest Godwin and co-worker's[1] recent description of the appearance of mycobacteria on Wright's-stained smears of peripheral blood and bone marrow, we report our experience observing mycobacteria in Wright's-stained preparations.

We also have seen mycobacteria in cytologic material from patients with acquired immune deficiency syndrome, including bone marrow aspirate smears and imprints, lymph node imprints, and peripheral blood smears. We agree substantially with the observations of Godwin and associates,[1] but in addition to the negative staining images they describe, we also have found refractile beaded bacilli, which, in our experience, often are easier to find than negatively stained bacilli.

Refractile organisms are seen both extracellularly and intracellularly. They also can be seen in crushed tissue debris or thick unsmeared marrow particles, which are inadequate for cytologic examination. Refractile organisms are easier to appreciate when the condenser is lowered. They are red if examined with microscopes with chromatic aberration or clear with apochromatic lenses. Acid fast stains remain the "gold standard" for the histologic diagnosis of mycobacterial infections.

We also agree with Godwin and associates[1] that recognition of mycobacteria using Wright's stain enables rapid identification of a serious and treatable infection. However, in our experience, only extremely rare organisms sometimes were found in peripheral smears examined retrospectively after finding many organisms in bone marrow preparations. We do not think that searching for these organisms in peripheral blood smears is a practical approach to the diagnosis of mycobacterial infection, although it is important to recognize that mycobacteria sometimes are found in peripheral smears.

EMINA TORLAKOVIC, M.D.
ELIZABETH D. AMES, M.D.
Department of Pathology
St. Luke's/Roosevelt Hospital
New York, New York

REFERENCE

1. Godwin JH, Stopeck A, Chang VT, Godwin TA. Mycobacteria in acquired immune deficiency syndrome. Am J Clin Pathol 1991;95:369–375.

NEWS AND NOTICES

Foundation, Department of Continuing Education, P.O. Box 94977, Cleveland, Ohio 44195; or call 800-762-8173 or 216-444-5696 (local calls).

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Foundation for Advanced Education in the Sciences

October 14–16, 1991   New Solutions to Old Problems in Diagnostic Pathology (Washington, D.C. area)

This three-day program will address difficult or evolving areas of diagnostic pathology and will focus on areas that often pose diagnostic difficulty. New techniques applicable to the general pathology laboratory will be presented, and clinicopathologic correlations of morphologic diagnoses will be stressed. Participants will receive a syllabus containing lecture outline material and references. The program is designed for pathologists at the senior resident and practitioner levels. Twenty-one hours of AMA Category I credit is available. For further information, write to Surgical Pathology, % FAES, One Cloister Court, Bethesda, Maryland 20814-1460; or call 301-496-7975.

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International Symposium on Automated Cytologic Screening for Cervical Cancer

October 17–19, 1991   Denver, Colorado

This meeting will offer in-depth analysis of developments in the field of automation in cytopathology and affords participants with the opportunity to become more aware of current methods of specimen preparation and video scanning technology. The main purpose of the symposium is to provide a forum in which the complex issues in this emerging field may be discussed. The directors are Heinz K. Grohs, M.D., Salem and Beth Israel Hospitals, Boston; and O. A. N. Husain, M.D., Charing Cross Hospital, London.

The three-day conference will include sessions on historical information of interest, analysis of market needs for automated screening, challenges for specimen standardization, issues of quality assurance, the possible role of the Federal Food and Drug Administration in this area, discussion of commercial applications of currently available technology, and workshops offering small group demonstrations of pertinent equipment. Participation is open to physicians, medical technologists, cytotechnologists, laboratory managers, and other interested members of the healthcare industry. For further information write to The International Institute for Applied CytoSciences, Inc., 40 Beach Street, Suite 304, Manchester, MA 01944; or call 508-526-8083; or Heinz K. Grohs, M.D., F.I.A.C., Program Chairman, 508-724-1200, ext. 4101.

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American Association of Pathologists

October 31–November 3, 1991   Concepts in Molecular Biology (Bethesda, Maryland)

Co-sponsored by the United States and Canadian Academy of Pathology and the College of American Pathologists, this course will consist of lectures, tutorials, laboratory demonstrations, and a syllabus with annotated bibliographies and laboratory protocols. The course has been designed for diagnostic and experimental pathologists, basic scientists, and clinical investigators who want to become conversant with the basic principles and concepts of recent advances in biotechnology. Emphasis will be placed on understanding nucleic acid molecular biology and its application to diagnosis and pathogenesis of human disease. Space is limited, so register early. Category I CME credit will be available. For more information and registration forms write to The American Asso-

ciation of Pathologists, 9650 Rockville Pike, Bethesda, MD 20814-3993; or call 301-530-7130, or fax 301-571-1879.

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Tulane University School of Medicine

November 8–9, 1991   Pathology Update (New Orleans, Louisiana)

This course is designed to provide the latest information on diagnostic approaches and new techniques in anatomic and clinical pathology. Topics include recent advances in surgical pathology, immunopathology, flow cytometry, diagnostic molecular pathology, TDM, hepatitis C, and early diagnosis of selected diseases. Invited speaker will be David L. Page, M.D., of Vanderbilt University. The course has been approved for Category I accreditation. Additional information may be obtained from the course directors, Terence T. Casey, M.D., and Michael A. Gerber, M.D., Department of Pathology, Tulane University School of Medicine, 1430 Tulane Avenue, New Orleans, Louisiana 70112; for information, call 504-588-5210.

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Department of Pathology, Massachusetts General Hospital, Harvard Medical School

November 18–22, 1991   Current Concepts in Surgical Pathology

This postgraduate course will be presented under the direction of Nancy L. Harris, M.D., Robert H. Young, M.D., and Eugene J. Mark, M.D.

This course is designed for pathologists at resident and practitioner levels. It will provide an in-depth review of diagnostic surgical pathology with emphasis on morphologic features, newly recognized entities, and new techniques, presented by the faculty of the Department of Pathology, Massachusetts General Hospital. Instruction will be primarily by lecture but also will include discussion periods. Each participant will receive a comprehensive course syllabus.

The course has Category I accreditation for approximately 35 hours of CME credit by the American Medical Association. The fee for the course is $650 (residents and fellows: $450). For further information, write to the Department of Continuing Education, Harvard Medical School, 25 Shattuck Street, Boston, MA 02115; or call 617-432-1525.

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The University of Utah

February 15–22, 1992   15th Annual Update in Clinical Microbiology and Immunology (Park City, Utah)

The University of Utah Department of Pathology, Division of Clinical Microbiology and Immunology, is pleased to offer this 25-hour review and update. Course directors are John M. Matsen, M.D., and Harry R. Hill, M.D. For further information write to Dr. Matsen, Department of Pathology, University of Utah School of Medicine, Salt Lake City, Utah 84132; or call 801-581-7480.

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Dermatopathology Symposium

February 15–23   Palm Springs, California

This symposium, taught by A. Bernard Ackerman, M.D., will consist of three 3-day courses: (1) A New Look at Histologic Diagnosis of Inflammatory Skin Diseases with Particular Emphasis on Superficial Perivascular Dermatitis, Vasculitis, and Panniculitis; (2) Mythology in Dermatopathology: Fictitious Diseases and Fallacious Concepts in Contemporary Dermatopathology; and (3) Still Another 100 Clues to Diagnosis in Dermatopathology (Clues III). Courses will run from 8:00 A.M. to 1:00 P.M. each day; breakfast sessions for residents will begin at 7:00 A.M. For further information, write to Florence Nygaard, 300 E. 40th Street, Suite 26H, New York, NY 10016; or call 212-263-7268.

INFORMATION FOR AUTHORS

Scientific Papers, Brief Scientific Reports, Case Reports

The *American Journal of Clinical Pathology* is devoted to prompt publication of original studies and observations in clinical and anatomic pathology. Original papers relating to laboratory use, management, and information science will be given consideration. An article is received with the understanding that it is submitted solely to the *American Journal of Clinical Pathology* and that no substantial portion of it is being submitted elsewhere. A manuscript based primarily on data published previously is not acceptable.

Send the original typewritten manuscript and two photocopies with three sets of illustrations to:

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Transmittal letter. Send with the manuscript a covering letter which states that 1) all authors have read and approve the final manuscript; 2) (if applicable) the authors will assume the cost of reproducing color illustrations; 3) when individuals (who have contributed significantly to the study) are acknowledged by name, their permission has been obtained to do so. Also, in conformance with the Copyright Revision Act of 1976, include the following statement, which has been signed by all authors (except federal employees): "In consideration of the *American Journal of Clinical Pathology* taking action in reviewing and editing the above named Article, the Author(s) hereby assign(s) to the American Society of Clinical Pathologists its legal representatives, successors and assigns all publishing rights and all other rights to the Article throughout the world, in any language and in any medium, including the right to procure the copyright thereon and the right to secure any renewals, reissues and extensions of any such copyrights in the United States or in any foreign country. Author(s) may request permission to reuse the manuscript or portions thereof. Such requests must be in writing and permission by the ASCP shall not be unreasonably withheld. The Author(s) appoint(s) the ASCP as the Author's(s') attorney-in-fact to execute any documents the ASCP deems necessary."

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Type manuscripts double-spaced throughout, with one-inch margins, on bond paper, 8½ × 11 inches. Begin a new page for each section (*i.e.,* title page, abstract, text, references, tables, legends, and acknowledgments), numbering pages consecutively.

Title page. Include on the title page: 1) a concise title; 2) first name, middle initial, last name of each author plus his highest degree; 3) institutional affiliation of each author; 4) name and address of author to whom reprint requests should be addressed; 5) acknowledgment of source(s) of support; 6) a brief title (40 characters total, including spaces); 7) disclaimers, if any.

Abstract and key words. The abstract, not exceeding 150 words, should state the study's purposes, procedures, and significant findings, with emphasis on new observations. Include 3 to 10 key words below the abstract.

Text. The *Introduction* should clearly state the purpose and rationale of the study being presented.

Materials and Methods. Established techniques may be referenced; however, new or modified methods should be described in sufficient detail to allow duplication of the study by an independent observer. Describe *Results* concisely and in a logical fashion. The *Discussion* should emphasize novel or significant aspects of the study; conclusions should not be based on unpublished observations or data derived solely from the previous literature.

Case Reports should not exceed four printed *journal* pages (about 12 double-spaced manuscript pages).

Nomenclature. Use current editions of *Dorland's Medical Dictionary* and *Webster's International Dictionary* as references. Use the nomenclature of bacteria given in *Bergey's Manual of Determinative Bacteriology.* Identify all drugs and chemicals by their generic names, followed, in parentheses, by the chemical formulas when deemed appropriate. The

TNM method for staging tumors is required (American Joint Committee on Cancer. *Manual for Staging of Cancer,* 2nd edition, J.B. Lippincott Co., Philadelphia, 1983).

Units. All quantitative measurements must be expressed in SI units, followed in parentheses, by the conventional metric units. pH, gas pressure measurements (pO_2 and pCO_2), and osmolality should be reported in conventional units only. Express temperature in degrees Celsius. Express enzyme activity in International Units per liter (U/L). Base all SI concentration units on a volume of 1 liter (symbol L). Express as amount of substance (mole) or mass (gram) units using appropriate prefixes, such as milli- or micro-. Conversions may be made using factors given in Am J Clin Pathol 1987; 87: 140–151. In describing reagent preparation, continue to give weights and volumes in conventional metric units only (*e.g.,* Stock 500 mmol/L glucose standard; add 0.900 g of glucose to 10 mL water in 100 mL volumetric flask, dissolve, and fill to the mark with water.).

Abbreviations. Use standard abbreviations whenever possible (see *Council for Biology Editors Style Manual,* Council for Biology Editors, 4th Edition, Arlington, VA, 1978). The full term for which the abbreviation stands, followed by the abbreviation in parenthesis, should precede the first use of the abbreviation in the text, except for standard units of measurement.

Human and animal experimentation. When reporting results of experimental investigations on human subjects, include a statement that the procedures followed were in accord with the ethical standards established by the institution in which the experiments were performed or are in accord with the Helsinki Declaration of 1975 (*Encyclopedia of Bioethics.* Vol 4, Warren T. Reich, Editor-in-Chief. Georgetown University, The Free Press, Division of Macmillan Publishing, New York, 1978, pp 1769–1773). Experimental investigations on animals must include a statement indicating that the institution's or the National Research Council's guide for the care and use of laboratory animals was followed.

References. References should include only articles cited in the text, and should be listed in order of citation. When there are four or fewer authors, list all of them. When there are five or more, list the first three and add "et al." Journal titles should be abbreviated according to *Index Medicus.* The following format should be used, and inclusive page references should be given.

1. Rosen PP, Kimmel M. Juvenile papillomatosis of the breast: a follow-up study of 42 patients biopsied before 1979. Am J Clin Pathol 1990;93:599–603.

Illustrations. Cite figures consecutively in the text, and submit them as sharp, glossy, black and white, unmounted photographs. All illustrations must fit the journal columns (3½ × ≤9 in. [single column]; 7 × ≤9 in. [double column]). Figures outside this range will be cropped or reduced at the editors' discretion. Art work and graphics must be of professional quality and legible. Indicate figure number and list first author on the top-back of all illustrations, by gummed labeling or writing lightly in pencil. Submit figure legends on a separate page, double-spaced. Stains and magnifications must be included. Photographs of persons should render them unidentifiable or include their written permission. All authors must assume the cost of reproducing color photographs.

Tables. Cite tables in the text in consecutive order. Type each table double-spaced on separate pages. Each column should contain a short heading. Place explanatory material (with nonstandard abbreviations, if not explained in the text) in footnotes to the table. Do not use internal horizontal and vertical lines.

Footnotes. Limit use of footnotes. Use symbols in the following order *, †, ‡, §, ∥, ¶, **, ††.

Disposition of manuscript. Receipt of manuscripts will be acknowledged and a decision regarding disposition made as soon as possible. Accepted manuscripts are subject to editorial revision and are not returned. Corresponding author will receive galley proofs for review. An order for reprints accompanies the galley proof.

Letters to the Editor. Only communications referring to an article recently published in the *Journal* are eligible for acceptance as *Letters to the Editor.* One month is allowed for the original author's reply, after which the letter(s) will usually be published within two months. However, for such rapid publication we cannot provide authors with galley proofs or reprints. *Letters to the Editor* containing figures or complicated tables will go through the usual process. Publication in that case takes about six months. In many instances, such letters are more appropriately submitted as a *Brief Scientific Report.*
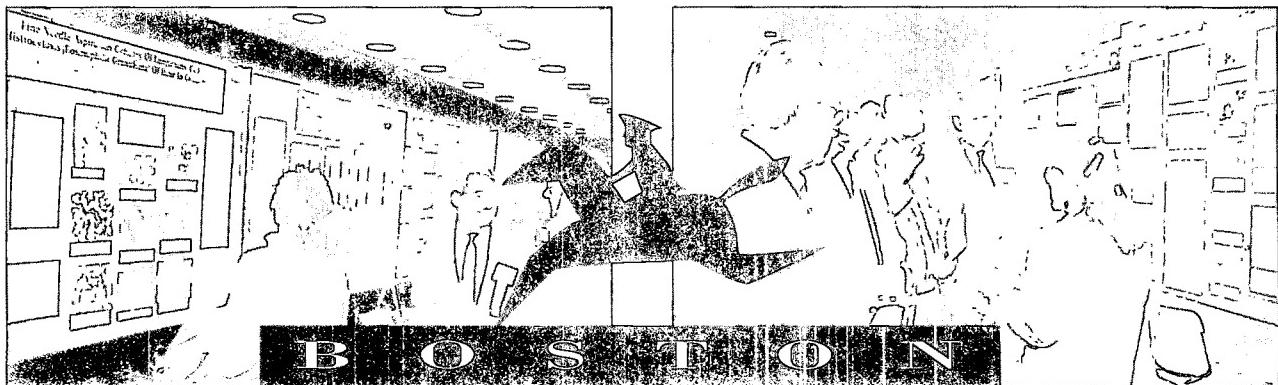
ASCP/CAP 1992 SPRING MEETING
APRIL 4-9

POSTER SESSIONS

All laboratory professionals are invited to participate in an exchange of the latest research in anatomic and clinical pathology. Scientific abstracts may now be submitted for presentation in a poster format at the ASCP/CAP 1992 Spring Meeting in Boston, Massachusetts. Abstracts are accepted on the basis of quality as determined by the ASCP Abstract Review Committee. Abstracts selected for poster presentation will be published and indexed in the *American Journal of Clinical Pathology*.

PATHOLOGY RESIDENT AWARDS

ASCP/CAP invite submission of abstracts from pathology residents for the Pathology Resident Awards. The sum of $500 each will be awarded to the authors presenting the best paper in anatomic pathology and the best paper in clinical pathology, respectively. Ten papers authored by residents, five each in anatomic pathology and clinical pathology, will be selected for presentation from the podium during the ASCP/CAP 1992 Spring Meeting. The abstracts of all ten finalists will be published and indexed by the *American Journal of Clinical Pathology*.

For information, call ASCP toll-free: 800-621-4142 (in Illinois, phone 312-738-4890). Or write: Manager for Scientific Assembly, American Society of Clinical Pathologists, 2100 West Harrison Street, Chicago, Illinois 60612-3798.

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Postmark deadline for submission is September 23, 1991.

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Academic Chemical Pathologist - The Pathology Department at Indiana University School of Medicine is seeking a Pathologist for an Assistant Professor tenure-track position in the Division of Clinical Pathology, and Board Certified or Eligible in Chemical Pathology, with fellowship training in Chemical Pathology or Clinical Chemistry. The position involves the professional direction of clinical laboratories as well as participation in the Division's educational programs. An interest and background in research is strongly recommended, with the candidate expected to pursue an independent research program. Research interests in protein/enzyme biochemistry, endocrinology, or medical informatics are highly desirable. The Department of Pathology at Indiana University Medical Center is a large academic department which directs clinical laboratories in four hospitals; has educational programs in medical technology, medical and graduate programs, as well as Residency and Fellowship trainingships. Send curriculum vitae and the names of three references to: Dr. Carleton D. Nordschow, Chairman, Department of Pathology, Indiana University Hospital, Room N-440, 926 West Michigan Street, Indianapolis, Indiana 46202-5283. Indiana University is an Equal Opportunity Employer.

CLEVELAND CLINIC FLORIDA - DEPARTMENT OF PATHOLOGY AND LABORATORY MEDICINE - Additional 1-2 pathologists sought to join a growing practice in the Department of Pathology/Laboratory Medicine at Cleveland Clinic Florida. Individuals must be board certified/eligible in anatomic/clinical pathology. Expertise required in surgical pathology/cytopathology and an area in clinical pathology. Preference given to candidates with demonstrated interest in potential academic development. Send curriculum vitae with names and addresses with three references to Fred V. Lucas, M.D., Department of Pathology & Laboratory Medicine, Cleveland Clinic Florida, Ft. Lauderdale, FL 33309. An equal opportunity/affirmative action employer.

TRANSFUSION MEDICINE DIRECTOR - PATHOLOGIST. Pathologist with interest and/or certification in Transfusion Medicine needed by a 556 bed acute care medical center with active Trauma, Cardiovascular Surgical and Oncology Services. Hospital is a principal teaching affiliate of the University of Illinois College of Medicine and is a participant in the Metropolitan Hospital Group Residency in Pathology. Blood Bank has donor, cytopheresis, and apheresis services and an annual workload of nearly 8,000 blood and 6,500 component transfusions. Candidate should be board eligible/certified and will also participate in surgical pathology, autopsy, and cytopathology rotations. Medical staff consists of nine board certified pathologists and three laboratorians with clinical interests. Inquiries and resumes to Douglas P. Rhone, M.D., Chief, Department of Pathology; Illinois Masonic Medical Center; 836 West Wellington Ave.; Chicago, Illinois 60657. Tele: (312) 296-7889.

Clinical Chemistry, Department of Pathology, University of Miami/Jackson Memorial Medical Center. The Department of Pathology at the University of Miami is recruiting a physician to serve as the Associate Director of Clinical Chemistry at the 1250 bed Jackson Memorial Hospital. We are seeking an individual who has experience in Clinical Chemistry and will work closely with the section Director in the development of clinical research and teaching programs and the implementation of administrative policies. The University of Miami Department of Pathology includes a faculty of 45 physicians and scientists and 19 house officers. The candidate for this position should be a BE/BC pathologist with experience in Clinical Chemistry. Appointment will be at the rank of Assistant or Associate Professor. Rank and compensation will be commensurate with demonstrated past accomplishments. Applicants should send their curriculum vitae and three references to: Gerald E. Byrne, Jr., M.D., University of Miami/Jackson Memorial Medical Center, Dept. of Pathology, 1611 N.W. 12th Ave., Miami, FL 33136.

BOARD CERTIFIED/ELIGIBLE CERTIFIED PATHOLOGIST - 1,004-bed GM&S and Psychiatry VA Medical Center is seeking pathologist with expertise in anatomical pathology and clinical laboratory medicine to direct laboratory staffed by 23 employees. The VAMC is affiliated with Pennsylvania State University College of Medicine, Hershey, Pennsylvania, and as such, has residents in medicine, surgery, urology, ophthalmology, anesthesiology and family medicine. Cost of living is reasonable and southcentral Pennsylvania is a delightful location. Salary and benefits are competitive. Contact Personnel Service (05), VA Medical Center, Lebanon, PA 17042. (717) 272-6621, ext. 4273. EOE.

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William M. Murphy, MD
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John S.J. Brooks, MD
October 18-20, 1991, Pasadena, California

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Immunohistology: Techniques and Interpretation for Immunoperoxidase, Immunofluorescence and In Situ Hybridization
David F. Keren, MD
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The Osler Institute
Pathology Review Course *honoring Paul Wolf*
November 9-16, 1991 – San Diego   May 24-31, 1992 – Indianapolis
Cytology Review Course *honoring Eileen King*
November 13-16, 1991 – San Diego   May 28-31, 1992 – Indianapolis

OBJECTIVES:
- To increase proficiency in anatomic and clinical pathology
- To assist pathology residents in organizing study
- To prepare candidates to take their board examinations
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Sun, Nov 10, 8am-10pm
General Pathology
- Injury and Repair
- Neoplasia
- Genetics
- Pediatric Pathology
- Forensic Pathology
- Electron Microscopy

M-Tu, Nov 11-12, 8am-9pm
Systemic Pathology
- Cardiovascular
- Pulmonary
- Gastrointestinal
- Endocrine
- Liver and Gallbladder
- Breast and Kidney
- Female Reproductive
- Male Reproductive
- Bone and Soft Tissue
- Dermatopathology
- Neuropathology

Wed, Nov 13, 8am-10pm
Cytology and Hematology
- Cytopathology
- Red and White Cells
- Platelets and Coagulation
- Lymph Nodes
- Lab Management

Thu, Nov 14, 8am-10pm
Immunopathology
- Humoral Immunity
- Cellular Immunity
- Immunodeficiency
- Hypersensitivity
- Autoimmunity
- Blood Banking

Fri, Nov 15, 8am-10pm
Microbiology
- Bacteriology
- Virology and Mycology
- Parasitology
- Sensitivity Testing

Sat, Nov 16, 8am-6pm
Chemistry
- Statistics and Q.C.
- Instrumentation
- Acid Base and Lytes
- Enzymes and Liver
- RIA and Toxicology
- Lipid and Protein
- Endocrinology
- Urinalysis

Sun, Nov 17, 8am-6pm
Extra Glass Slide Lab
- Robert Astarita, M.D.
- Colin Bloor, M.D.
- Delver Cain, M.D.
- Jeffrey Cao, M.D.
- P. Chandrasoma, M.D.
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- Nancy Warner, M.D.
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- Edward Wong, M.D.

Cytology Review

Fri-Mon, Nov 13-16
Exofoliative Cytology
Aspiration Cytology

- Robert Astarita, M.D.
- Carol Carriere, C.T.
- P. Chandrasoma, M.D.
- Tina Fanning, M.D.
- Darryl Heustis, M.D.
- Eileen King, M.D.
- Fritz Lin, M.D.
- Shahla Masood, M.D.
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